



UNIVERSITÀ DEGLI STUDI DI MILANO



Maastricht University



MERLN Institute

Cotutelle PhD Thesis

THE MANY FACES OF REGENERATION

ENGINEERING MULTIFUNCTIONAL SCAFFOLDS FOR CRANIOMAXILLOFACIAL APPLICATIONS

PhD in Translational Medicine
Biomedical Sciences for Health Department
XXXVIII cycle

Institute for Technology-Inspired Regenerative
Medicine (MERLN)
Complex Tissue Regeneration Department

PhD Candidate
Sophia Dalfino
Matr. R13817
ORCID n. 0000-0003-2165-0582

Supervisors
Dr. Claudia Dolci (UniMi)
Prof. Gianluca Tartaglia (UniMi)
Prof. Lorenzo Moroni (UM)

Academic Year 2024 - 2025

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CHAPTER 1

General introduction

Clinical problems and standard treatments

A mandibular bone defect refers to a volume of the lower jaw where bone is missing. These defects have a significant impact; they not only interfere with chewing, speaking, and non-verbal communication, but also impact the aesthetic of the face. Thus, the quality of life of individuals affected by such defects, including their physiological well-being, is highly impaired [1].

Mandibular defects arise from congenital deformities, traumas or tumor resections, with the latter often leading to larger and more complex defects. Head and neck squamous cell carcinoma (HNSCC) rank 7th among the most common global cancers, with around 900,000 new patients affected and 450,000 deaths every year [2]. Of these, 40% involve the oral cavity, equaling approximately 378,000 annual new cases [2]. Moreover, the incidence rate of HNSCC involving the oral cavity has been increasing in many countries, with a predicted 30% increase in incidence rate by 2030 [3].

To restore the mandible after tumor resections, surgeons need to reconstruct the missing parts. Historically (i.e. early post-war procedures), the reconstruction procedures were based on non-vascularized bone grafts and metal implants, which had limited success in replacing the defect, especially for bigger resections. An alternative procedure rose only in 1989, when Hidalgo et al. described for the first time the use of a vascularized fibular bone flap for mandible reconstruction [4]. This procedure involves transplanting a section of the patient's fibula (leg bone), including blood vessels, between the two bone margins to reconnect the mandible. The graft is then stabilized with metal fixation plates, and finally micro-anastomoses are made to connect blood vessels [5]. This procedure remains up to this day the gold standard replacement technique.

Despite the high success rate of 90% [3], the current treatment is associated with highly significant limitations. The surgical procedure is complex, involves long operating times, and two surgical teams: one resecting the mandible and one harvesting the fibula. Advanced microsurgical skills are required to precisely anastomose blood vessels in the jaw. This complexity increases the overall surgical risk [6]. Foremost is donor-site morbidity, which refers to long-term effects and complications that occur in the leg. Up to approximately 30% of the patients experienced complications such as infection (1-10%), skin graft failure (8-15%), and wound re-opening (7-8%) [7,8]. Chronic pain and impaired walking capacity are also reported as consequence of removing part of the fibula, impairing the patient's quality of life. On the other hand, at the recipient site in the jaw, differences in bone composition (e.g. bone density) or size (e.g. bone diameter or vertical height) between the fibula and the native mandible lead to mechanical and anatomical mismatches. These can complicate post-surgery rehabilitation procedures, such as implantation of dental prosthetics, by decreasing stability and compromising functionality

[9]. Additionally, the fibula is a straight bone, while the mandible presents curved anatomy, making it challenging to restore function and aesthetics.

Taken together, there is a pressing need to overcome these challenges to improve patients' outcome in the long-term. In this context, we understand why, particularly from 2008-2010, research efforts for mandibular reconstruction shifted towards bone tissue engineering (BTE) and bone-inducing biomaterial-based approaches, which are emerging as promising alternatives to overcome conventional treatments limitations [10].

Bone tissue engineering

BTE is an interdisciplinary field that combines engineering, material and biological sciences. Here, tissue engineers design 3D constructs (i.e. scaffolds) that mimic biological, functional, and mechanical features of the native tissue, serving as a temporary support for cells. The properties of the constructs are designed in a way to gain control over the behavior of the hosted cells and instruct them to deposit novel bone tissue after implantation. Cells can infiltrate these scaffolds either via *in vitro* seeding procedures or *in situ* from the tissue surrounding the implantation sites. The newly formed bone needs to possess appropriate structure and composition to restore correct tissue functionality.

BTE offers a promising alternative to traditional autologous grafts – avoiding limitation such as low availability of tissue that can be harvested and invasiveness – as they can be produced in an off-the-shelf manner. They rely on the body's natural regenerative capacity to heal and integrate the construct. Tissue engineers use biodegradable materials – synthetic or derived from natural resources – to create 3D constructs. Over time, while the tissue is being regenerated, the scaffold is gradually degraded by the body. Here, the properties of the material are a crucial aspect as they directly influence the behavior of infiltrating cells. Particularly, materials for bone applications need to be osteoinductive (able of inducing differentiation of progenitor cells into osteoblasts) and promote osteointegration (structural and functional connection between the implant and the surrounding bone tissue). Materials commonly used in BTE are inorganic phosphate ceramics and bioactive glasses, which not only possess bioactivity, but also closely resemble the natural bone composition, which is made of a 60-70% inorganic mineral content, primarily in the form of hydroxyapatite.

The emerging of additive manufacturing (AM) techniques, such as melt-extrusion printing, advanced the field of BTE. They allow the processing of a broad range of biomaterials into 3D porous structures, in a layer-by-layer manner, with high control over scaffold geometry and pore architecture. Moreover, the potential combination of AM with computer aided design or manufacturing (CAD/CAM) techniques permits patient-specific customization of the shape of an implant.

Despite the many *in vitro* and *in vivo* studies proposing AM scaffolds for mandibular bone regeneration, their clinical translation remains limited. A major challenge, particularly in large defect models, is the insufficient amount of formed bone to fully restore the tissue functionality. In this context, this thesis aims at critically assessing the state of art, identifying the main limitations, and proposing new strategies to enhance bone regeneration, providing new insights that could facilitate clinical translation in the future.

Engineering mandibular bone: multiple approaches

In **Chapter 2**, we reviewed the state of the art of tissue engineering approaches for mandible regeneration using 3D scaffolds, with a focus on studies that have reached the preclinical (i.e. *in vivo*) phase. We initially outlined the key scaffold requirements – including material composition and pore geometry – and reported different strategies to enhance osteogenesis and vascularization. Then, we quantitatively analyzed and compared *in vivo* bone regeneration outcomes, such as bone volume and bone density, in relation to scaffold design parameters and integrated biological cues. The results were also discussed in perspective of the defect model itself, highlighting differences between small and large animals, which are crucial for interpreting preclinical results and their potential clinical translation. As main challenges we identified the ability of the constructs to induce high levels of bone formation and sufficient vascularization to support tissue regeneration after implantation.

Thus, in the experimental part of this thesis, we focused on addressing the limitations of existing mandibular bone scaffolds by investigating different approaches and proposing novel strategies. Four strategies are mainly investigated to engineer mandibular bone: 1) designing complex pore geometries to enhance osteogenic differentiation, 2) introducing bioactive materials to promote osteogenic and angiogenic scaffold properties, 3) fabricating bioactive coatings on scaffold fibers to simultaneously induce osteogenesis and release pro-angiogenic compounds, and 4) developing a multicomponent hybrid scaffold to achieve *in vitro* construct pre-vascularization (**Figure 1**).

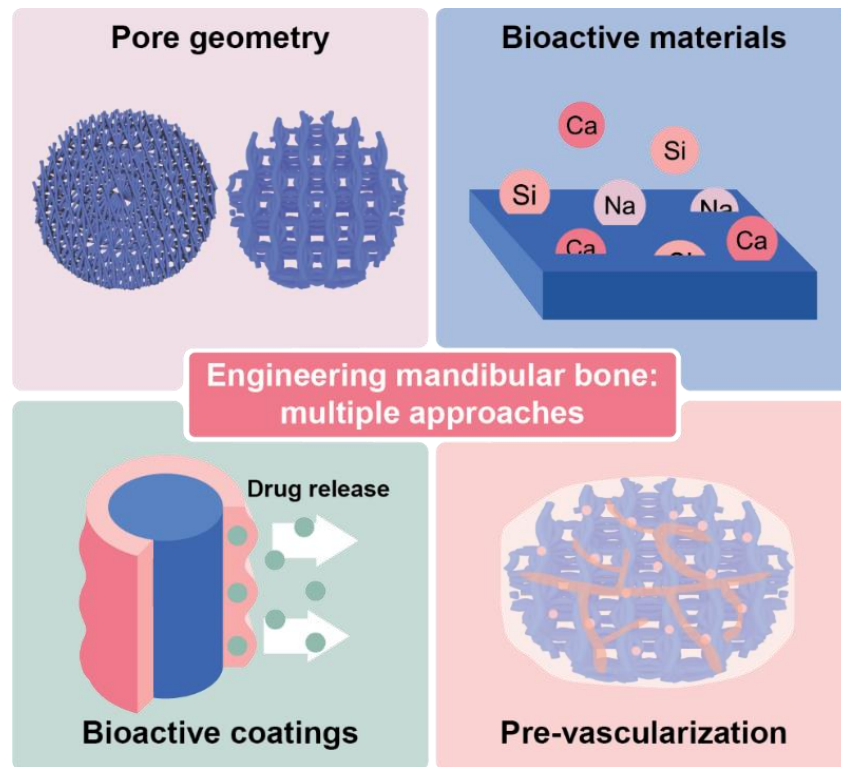


Figure 1. Schematics of the thesis outline, showing different approaches investigated to engineer mandibular bone: the effect of scaffold pore geometry, incorporation of bioactive materials, implementation of bioactive coatings and construct *in vitro* pre-vascularization.

First, in **Chapter 3**, we explore the role of scaffold pore geometry in osteogenesis. We design scaffolds with star and diamond pore morphologies, inspired by the native bone architecture. Both complex structures, fabricated with AM, comprise of polycaprolactone (PCL) and a high content (i.e. 40%) of β -tricalcium phosphate (β -TCP), a ceramic material that has osteoinductive properties. Then, we assess their impact on the differentiation and matrix production of human mesenchymal stromal cells (hMSCs) *in vitro*. Our findings demonstrate that both pore geometries support osteogenesis, with the diamond-shaped pores enhancing osteogenic gene expressions, and the star-shaped pores promoting matrix mineralization. This chapter highlights the importance of pore design as a geometrical parameter for mandibular BTE.

As further discussed in Chapter 2, angiogenesis plays a crucial role in bone regeneration and is currently a limiting factor in clinical translation. Therefore, in the following chapters described in this thesis, we focus on the design of scaffolds with not only osteogenic, but also angiogenic properties. In **Chapter 4**, we describe the development of composite scaffolds comprising PCL and two novel formulations of bioactive glasses (BGs), a class of ceramic materials known for their osteogenic and angiogenic properties resulting from their ionic composition. We evaluate the *in vitro* response of hMSCs and human umbilical vein endothelial cells (hUVECs) in relation to the chemical composition of the BGs. We showcase that BG scaffolds can successfully induce upregulation of osteogenic and angiogenic markers in hMSCs and improve tubule formation capacity of hUVECs. These results confirm

that we can take advantage of intrinsic material properties to induce favorable biological outcomes, supporting the potential of BG scaffolds for bone regeneration and vascularization.

In Chapter 3 & 4, the bioactive elements are incorporated in the bulk of the 3D fibers via a blending approach. The advantage of such an approach is that the biological cues (i.e. ions) are slowly released over time, while the synthetic component of the scaffold degrades. To achieve a faster release and a quicker biological effect, in **Chapter 5** we present a surface modification strategy. We describe a scaffold with a protein-based coating (i.e. elastin-like recombinamer (ELR)) that induces biomineralization directly on the surface of the fibers. Importantly, we load the coating with two pro-angiogenic molecules, either deferoxamine or phenanthroline, to induce a hypoxic-like response in hMSCs upon release. We observe an induction of pro-angiogenic genes at specific drug concentration thresholds. Simultaneously, we demonstrate the upregulation of osteogenic genes. Our results demonstrate the close interplay between angiogenic and osteogenic cascade that can be activated by modified scaffold surfaces within only a few hours after cell seeding.

The last approach to engineer mandibular scaffolds is presented in **Chapter 6**. Here, we describe the formation of a pre-vascularized construct comprising out of a stiff and soft component. We embed PCL/ β -TCP diamond-shaped scaffolds in a custom-made hyaluronic acid hydrogel, functionalized with peptides with various bioactive properties. We assess the potential of hybrid constructs to support microvessel formation and simultaneous osteogenic differentiation when a co-culture of hMSCs and hUVECs is encapsulated. We demonstrate that our constructs can induce tubule-like organization of hUVECs and expression of pericyte-related gene in hMSCs. These findings indicate that our hybrid system may support *in vitro* pre-vascularization, a promising strategy to facilitate implant integration *in vivo*.

In **Chapter 7** we discuss the main findings described in this thesis in perspective of the state of the art. We also speculate on the remaining limitations involved in clinical translation of these types of constructs and suggest future directions for the field. Finally, in **Chapter 8**, we present the societal, scientific and economic impact of the work described in this thesis.

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CHAPTER 2

Regeneration of critical-sized mandibular defects using 3D-printed composite scaffolds: a quantitative evaluation of new bone formation in *in vivo* studies

Sophia Dalfino, Paolo Savadori, Marco Piazzoni, Stephen Thaddeus Connelly, Aldo Bruno Gianni, Massimo Del Fabbro, Gianluca Martino Tartaglia, Claudia Dolci, Lorenzo Moroni

Adapted from Dalfino et al., *Advanced Healthcare Materials*, 2023,
10.1002/adhm.202300128.

Abstract

Mandibular tissue engineering aims to develop synthetic substitutes for the regeneration of critical size defects (CSD) caused by a variety of events, including tumor surgery and post-traumatic resections. Currently, the gold standard clinical treatment of mandibular resections (i.e. autologous fibular flap) has many drawbacks, driving research efforts towards scaffold design and fabrication by additive manufacturing (AM) techniques. Once implanted, the scaffold acts as a support for native tissue and facilitates processes that contribute to its regeneration, such as cells infiltration, matrix deposition and angiogenesis. However, to fulfil these functions, scaffolds must provide bioactivity by mimicking natural properties of the mandible in terms of structure, composition and mechanical behaviour. This review aims to present the state of the art of scaffolds made with AM techniques that are specifically employed in mandibular tissue engineering applications. Biomaterials chemical composition and scaffold structural properties are deeply discussed, along with strategies to promote osteogenesis (i.e., delivery of biomolecules, incorporation of stem cells, and approaches to induce vascularization in the constructs). Finally, a comparison of *in vivo* studies is made by taking into consideration the amount of new bone formation, the CSD dimensions and the animal model.

Keywords

Bone tissue engineering, Mandible, Maxillofacial, Polymer, Composite, 3D printing

Introduction

Mandibular bone defects may be attributed to different causes. Small defects commonly originate from tooth extraction or minor traumas, while larger ones result from severe traumatic injuries, degenerative diseases, congenital disorders, and tumour resections [1]. If the dimensions of the defects are below a critical size, the bone tissue can restore the damage through physiological healing processes [2]. Commercial products such as Boneceramic™ (Straumann®), Grafton® DBM (Biohorizons®), and Bonalive® which are granule-based synthetic materials, can further enhance this regeneration. Larger defects, exceeding the critical size (CSD), cannot be spontaneously repaired by the body, leading to the formation of fibrotic tissue instead of mineralized healthy bone [3].

The concept of a CSD was first defined by Schmitz & Hollinger (1986) as “*the smallest size intra-osseous wound in a particular bone and species that will not heal spontaneously during the lifetime of the animal*” [4]. This definition was later modified as: “*the size of a defect that will not heal over the duration of the study*”, to accommodate the limited duration of most of the studies [5]. These defects are particularly challenging for the maxillofacial region, which is anatomically complex and has several functions such as chewing, talking, swallowing, and breathing. The mandible also significantly differs from long bones for its fast-remodelling kinetics and embryonic development similar to craniofacial bones. It originates from the neural crest cells of the neuroectoderm germ layer, and it undergoes intramembranous rather than endochondral ossification. Moreover, mandibular bone marrow-derived stem cells exhibit higher osteogenic potential compared to other skeletal bones [6]. Thus, tissue engineering strategies designed for long bones are not directly translatable to mandibular regeneration.

Current strategies for mandibular bone reconstruction rely on bone autografts, allografts, or xenografts, which support healing through three important processes: osteoinduction (i.e. differentiation of progenitor cells into osteoblasts), osteoconduction (i.e. bone tissue growth), and osseointegration (i.e. graft integration in the surrounding native bone) [7-9]. The fibular flap autograft, which was first introduced by Hidalgo in 1989 [10], remains the gold standard for mandible reconstruction. This technique consists in harvesting a portion of the fibula and fixing it within the mandibular defect using metallic plates to ensure mechanical support [10, 11]. Those plates can be hand-bent or specifically shaped on the patient CSD, thanks to modern computer-aided design (CAD) and computer-aided manufacturing (CAM) technologies. Autografts do not induce immune rejection, and contain natural osteogenic molecules, such as bone morphogenic proteins (BMPs) and platelet-derived growth factor (PDGF), which ensure osteogenic pathways ignition [12, 13]. Nonetheless, the surgical procedure remains invasive, requiring at least two surgeries, which can result in patient discomfort, morbidity, and failure of the graft integration [9]. Finally, post-operative care is crucial to ensure functional recovery from the surgery,

preventing suboptimal tissue performance and altered facial physiognomy, thus compromising patient's quality of life [14, 15].

The limitations of the current clinical approaches are driving mandibular bone tissue engineering (BTE) towards the use of synthetic substitutes as alternatives to biological grafts. In this context, additive manufacturing (AM) is considered the most promising technique in tissue engineering to fabricate advanced materials as scaffolds [1]. The reasons are multiple: rapid prototyping, high repeatability, low production costs, free-form fabrication, and patient-specific fidelity [16]. Imaging technologies such as computed tomography (CT) and magnetic resonance imaging (MRI) enable precise modelling of the defect, which is then converted into a printable CAD model (**Figure 1**).

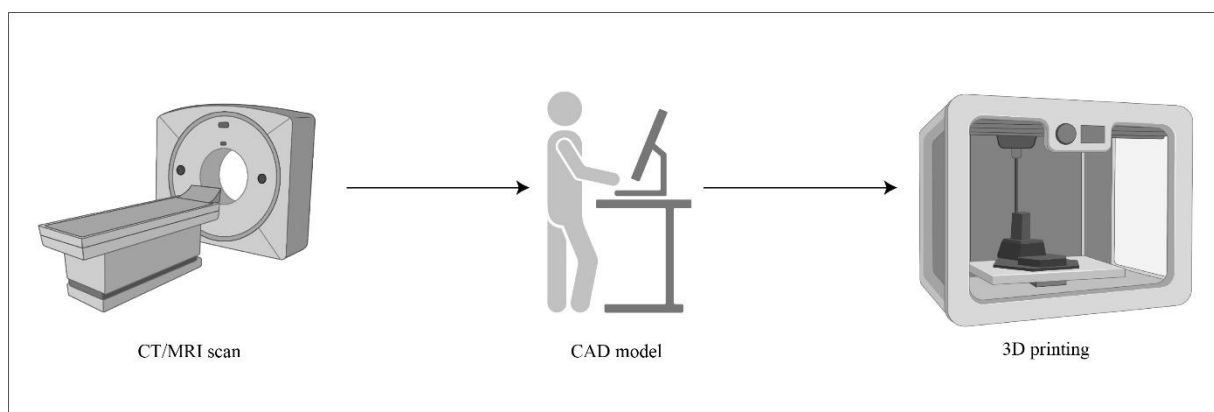


Figure 1. Steps of the scaffold printing process. Patient's defect is obtained through CT or MRI images; a virtual 3D model of the scaffold is created with CAD software; the scaffold is manufactured using a 3D printer. Created with BioRender.com.

Scaffolds produced via AM should mimic the mandibular tissue in terms of macro- and microstructure, composition and function. Indeed, a scaffold has to be porous with interconnected pores to favour mass transport of oxygen and nutrients into the inner core of the construct, to allow homogeneous cell colonization, and support vascularization [17-19]. Higher values of porosity and pore diameter, in general, correlate with greater bone formation and superior vascularization [20, 21].

An advantage of using AM techniques is the possibility to print several classes of biomaterials [22, 23]. However, those employed in mandibular BTE are essentially limited to calcium phosphate ceramics and synthetic polymers. The first category includes hydroxyapatite (HA) and β -tricalcium phosphates (β -TCP), whereas the second one, poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(lactide-co-glycolic acid) (PLGA), poly(caprolactone) (PCL), and photocurable polymers (i.e. resins). Both ceramics and polymers can be printed with different AM techniques such as vat photopolymerization (i.e. stereolithography (SLA) and digital light processing (DLP)), selective laser sintering (SLS) and fused deposition modelling (FDM) [24].

In addition, to tune biomaterials chemical composition and structure parameters, many studies demonstrate that including biological elements in the construct can boost mandibular bone reconstruction much further [25]. Of marked relevance there are osteoinductive bioactive molecules (e.g. BMPs) and growth factors that promotes the healing process (e.g. PDGF). They can be encapsulated in specific delivery systems (e.g. hydrogels) to achieve simultaneously spatial- and time-controlled release from the scaffold, and prevent heterotopic bone formation due to high dose-related side effects [25]. Cells like mesenchymal stromal cells (MSC) and endothelial cells can also be added to stimulate new bone formation and vascularization within the defect area [26]. Additionally, the scaffold can be prefabricated in a living bioreactor (i.e. a heavily vascularized tissue of the body) prior implantation in the mandibular bone, to establish a mature capillary network [27].

This review aims to illustrate the state of the art in the specific field of mandibular bone CSDs regeneration using polymeric or composite scaffolds fabricated through AM techniques. To the best of our knowledge, most of the reviews found in literature address the bone regeneration topic focusing on long bones; although, as previously discussed, the mandibular bone presents peculiarities that makes its reconstruction a more challenging clinical reality. Moreover, this review differs from others addressing the field of mandibular BTE. Park *et al.* [28] recently reported an analysis of the on-demand 3D bio-printing methods for the reconstruction of mandibular defects, whereas Nyirjesy *et al.* [29] investigated the history and evolution of 3D printing for head and neck oncologic surgery and bone reconstruction. The main goal of our work is instead to highlight the best scaffold features to ensure optimal mandibular bone regeneration. In particular, we will discuss materials chemical composition, AM techniques, structural and design parameters, bioactive molecules, vascularization strategies and cell types. At the end, mandibular BTE strategies will be quantitatively evaluated in terms of new bone formation data obtained from *in vivo* studies, thus facilitating an objective and straightforward comparison among different experimental approaches, CSD dimensions and animal models.

Research strategy

In order to find articles pertinent to this review, PubMed and Scopus electronic databases were searched using the following search strategy: (scaffold) AND (mandible[Title/abstract] OR jaw[Title/abstract] OR mandibular[Title/abstract]) AND (3d printing OR 3d printed OR three dimensional printed OR three dimensional printing OR additive manufacturing) AND (polymer OR composite OR polymeric). Furthermore, only articles published in the last decade (from 2012 to 2022) in English language were considered. No limitation regarding study design was set. A total of 23 articles were selected (**Table 1**).

Table 1. Overview of reviewed articles. Column headers represent: (i) material chemical composition, (ii) scaffold structural properties (i.e. pore size and overall porosity), (iii) biologically active molecules, (iv) cells added in the constructs, (v) size of the animal model used for *in vivo* experiments, and (vi) main results in terms of new bone formation.

Materials	Structure	Biomolecules	Cells	Animal model	Results	Ref.
HA/PLGA	Ø=480-520 µm	-	-	Small	BV/TV~25% MV/TV~45% At 8 weeks	[30]
Lay-Fomm 60®	Ø=750 µm (macropores) Ø=0.2-20 µm (micropores)	-	DPSCs	Small	BV/TV=30.26±9.4 6%	[31]
PLGA/nHA	Porosity=74% Ø=430 µm	rhBMP-2	-	Small	BV/TV~25% at 8 weeks	[32]
PCL/TCP	NA	-	hAFSCs	-	Cell viability=91±2% after 1 day in the printed bone	[33]
β-TCP/PCL	Ø ₁ =5-40 µm Ø ₂ =7-300 µm	-	pBMPCs	Big	Bone PSA=22.11±22.45	[34]
PCL/TCP/ bdECM	NA	-	ADSCs	Big	NBV=372.32 HU after 8 weeks	[35]
PCL/β-TCP	Porosity=57% Ø ₁ =300 µm Ø ₂ =600 µm	-	-	Big	BV/TV~13% after 12 weeks	[36]
β-TCP	Ø=330 µm	Dipyridamole	-	Small	BV/TV~25% after 8 weeks	[37]
PLA	Ø=50-300 µm	nHA Carbon nanotube	C3H/10 T1/2	-	Cell viability=150% from MTT assay at 7 days	[38]
PCL/β-TCP	Porosity=50% Ø=500 µm	rhBMP 2	MSCs	Big	BV/TV=48.443±0. 25% after 12 weeks	[39]

PCL/ β -TCP	NA	-	TMSCs	Small	BV/TV=57.44% after 12 weeks	[40]
PCL/ β -TCP/hydrogel	NA	Resveratrol Strontium ranelate	MSCs Osteoclasts HUVECs	Small	BV/TV~25% after 8 weeks	[41]
PLGA/ β -TCP	Porosity=63.7% $\varnothing$ =358 μ m	rhBMP-2	-	Big	BV/TV~20% after 3 months	[42]
PCL/HA	Porosity=83.3% $\varnothing$ =470 μ m	-	MC3T3-E1	-	Cell proliferation assessed at 7 days	[43]
Magnesium substituted calcium silicate scaffolds	Porosity=58% $\varnothing$ =480 μ m $\varnothing$ =600 μ m $\varnothing$ =720 μ m	Ions	-	Small	BV/TV~25% at 12 weeks	[44]
HA/TCP	NA	rhBMP-2	-	Big	BV/TV~40% after 12 weeks	[45]
β -TCP	Porosity=64%	-	-	Big	After 6 months CT data confirmed osseointegration	[46]
PCL/ β -TCP	Porosity=57%	rhBMP-2	MC3T3-E1	Big	NBV=10.08 $\pm$ 2.48 mm ³	[47]
PCL	Porosity=20% - 80%	-	ADSCs	Small	In vivo assessment of vascularization after 7 days	[48]
PEEK	Porosity=50% $\varnothing$ =750 μ m	-	ADSCs	Small	BV/TV=61.27% $\pm$ 8.24 at 20 weeks	[49]
PLA	Porosity=85%	BMP-2	-	Big	BV/TV=25-32% after 3 months	[50]
PGLA/HA	$\varnothing$ =400 μ m	-	-	Small	MV/TV=9.88 $\pm$ 4.6% at 4 weeks	[51]
PCL/nHA	Porosity=53.53 %	-	-	Big	Histologic results confirmed angiogenesis and bone formation	[52]

NA = not available; BV/TV = bone volume/total volume; PSA = percentage surface area; HU = Hounsfield unit; NBV = new bone volume; MV/TV = mineral volume/total volume; DPSCs = Dental Pulp Stem Cells; hAFSCs = Human Amniotic Fluid Stem Cells; pBMPCs = Peripheral Blood Mononuclear Cells; ADSCs = Adipose Tissue-derived Stem Cells; C3H/10T1/2 = cell line from C3H mouse embryo cells; MSCs = Mesenchymal Stem Cells; HUVECs = Human umbilical vein endothelial cells; MC3T3-E1 = immature osteoblast murine cell line.

Biomaterials for mandibular regeneration

Similarly to long bones, the mandible can be defined as a composite material consisting of a mineral phase of calcium phosphates, an organic phase of collagen type I and non-collagenous proteins, and water. The amount of each part may vary depending on the

patient's age, location, gender, ethnicity, and medical conditions [53]. Imamura *et al.* recently compared the micro-structure and bone composition of human mandibular specimens, harvested from the symphysis and the ramus, to the tibia and ilium (**Figure 2**) [54].

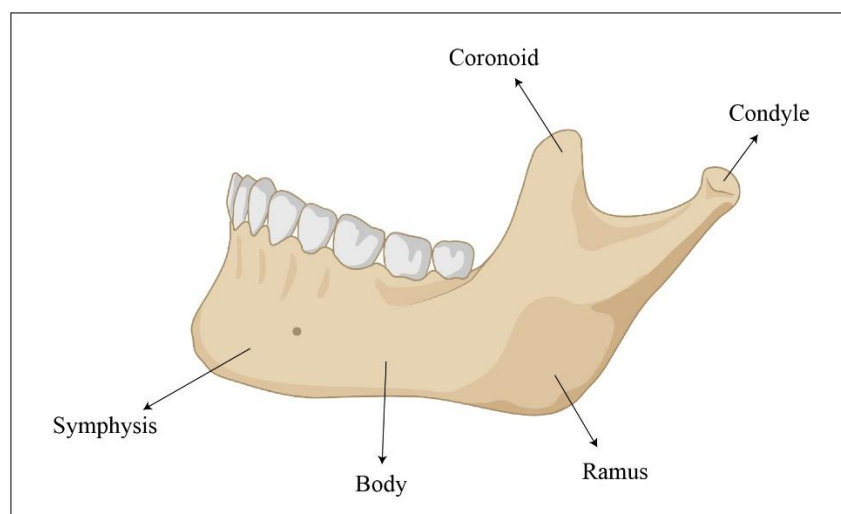


Figure 2. Anatomy of the mandible. Created with BioRender.com.

Using micro-computed tomography (μ CT), they measured several parameters, such as the percentage fraction of the bone volume over the total volume (BV/TV%) and the bone mineral density (BMD), defined as the volume density of calcium hydroxyapatite ($\text{mg}\cdot\text{cm}^{-3}$). Higher values of BV/TV% were revealed in the mandibular samples compared to those extracted from tibia and ilium (**Table 2**). These results suggested that different bone sites are characterized by compositional and structural differences. This feature should be considered for an accurate evaluation of the *in vivo* results.

Table 2. Comparison of BMD and BV/TV in four different sites [54].

	Symphysis	Ramus	Tibia	Ilium
BV/TV [%]	34.4 $\pm$ 13.7	30.1 $\pm$ 9.2	18.1 $\pm$ 6.7	22.0 $\pm$ 8.8
BMD [$\text{mg}\cdot\text{cm}^{-3}$]	1030.9 $\pm$ 168.3	859.7 $\pm$ 130.5	813.1 $\pm$ 115.0	756.6 $\pm$ 112.3

To achieve good results, the ideal scaffold should be designed to mimic the microstructure, and composition of natural bones as closely as possible, which involves selecting appropriate biomaterials. The most commonly used biomaterials for the production of scaffolds for mandibular tissue regeneration are synthetic polymers (e.g. PCL, PLA and PLGA) and ceramics (TCP and HA) (**Table 1**). These two classes of materials can both be processed through AM techniques.

Synthetic polymers are synthesized under controlled and reproducible conditions, offer good mechanical properties, and are generally biodegradable and biocompatible. Their degradation time varies from a few months to a few years, depending on the exact chemical composition and molecular weight. It is also possible to control the impurities present in the material, reducing the risk of toxicity and infection [55]. PLA is a thermoplastic polyester that has been used for various medical implants, including bone screws, fixation devices and vascular grafts, due to its biocompatibility, degradability, high mechanical strength and low immunogenicity [18, 38]. Manjunath *et al.* reported FDM-fabricated PLA scaffolds, embedded with a PCL matrix to release biomolecules into the external environment [38]. In addition, a clinical-grade PLA scaffolds coated with a polyelectrolyte was engineered to release BMP-2 proteins [50]. In both cases, the PLA scaffolds acted as the structural component, providing the shape of the anatomical part to be regenerated.

PCL has been extensively studied for tissue engineering, due to its physico-chemical properties, such as mechanical strength, prolonged biodegradation (almost three years for complete removal from the body for PCL with average MW = 50 kDa) and biocompatibility [34, 38, 55]. The high mechanical strength and slow degradation rate of PCL allow for load bearing properties during gradual formation of new bone tissue. This is an important property, since mandibular bone has to face high loads during the patient's life. Temple *et al.* and Zamani *et al.* confirmed the successful use of PCL alone to print structures to support mandible tissue regeneration [48, 56]. PLGA is a synthetic copolymer of PLA and PGA. It has excellent degradability, which can be adjusted according to the ratio of PLA to PGA, and biocompatibility, and is widely used as a drug carrier material [32].

Despite their good mechanical properties and biocompatibility, synthetic polymers usually have limited cell adhesion properties, and lack of bioactivity (i.e. the ability of a material to induce and accelerates the mineralization on its surface) [57]. Therefore, they can be combined with bioceramics that are generally osteoinductive and osteoconductive [55, 58]. The most commonly used bioceramics for mandible regeneration are calcium phosphates, because they are already present in high amounts in the native bone tissue [18]. These materials have excellent osteoinductivity and osseointegration properties because of their chemical composition [59]. The release of Ca^{2+} and PO_4^{3-} ions in the surrounding environment sure enough is a signal that guide cell migration, bone remodelling, and matrix mineralization [59, 60]. Phosphate ceramics exhibit, in general, lower toughness compared to the natural cortical bone and this restricts their use to non-load bearing applications [61]. Nevertheless, it is worth noting that mechanical strength depends on particle size and shape, and it can be tuned by mixing different ceramics or performing treatment on the ceramic powders [61, 62]. HA is the main inorganic component of bone tissue and for this reason has intrinsic excellent properties for

mandibular BTE, like high biocompatibility, no cytotoxicity, long-term biodegradability. This last feature, can be also tuned by sintering temperature, porosity and pore diameter [63].

The optimal osteoconductive properties allow HA scaffolds to form a strong bond with the surrounding bone [18]. HA has been shown to induce the proliferation and osteogenic differentiation of human bone marrow-derived MSCs *in vitro*, as it is able to promote the expression of osteogenic growth factors, such as BMP and alkaline phosphatase (ALP) [18, 30, 32]. Therefore, Ciocca *et al.* functionalized PCL scaffolds with nHA to increase hydrophilicity and enhance cell adhesion and differentiation [52]. Both Deng *et al.* and Chang *et al.* reported that combining HA and PLGA can increase the bioactivity of the polymer [30, 32]. Interestingly, Chang *et al.* printed a hyperelastic biomaterial (HB) made of 90% of HA and 10% of PLGA to evaluate its osteoregenerative ability [51]. Such a high amount of HA present in the composite material should enhance the bioactivity of the scaffold, slow down polymer degradation rate as well as the amount of by-product derived from it.

Another category of ceramics is β -tricalcium phosphate (β -TCP), which has a faster resorption compared to HA [34]. In addition, β -TCP has a high affinity for BMP-2, which is a key growth factor required for bone production. Because of these characteristics, β -TCP can lead to a rapid bone formation straight after implantation, improving the regeneration of mandibular bone tissue. This is why it is often used in combination with polymers to create composite scaffolds [35]. A composite scaffold based on PCL and β -TCP was tested in several studies (**Table 3**), with different ratios of the two components, showing promising results in different animal models. Composite scaffolds of 50/50 (%w/w) PCL/ β -TCP, pre-seeded with porcine MSCs, induced a good depth of bone penetration in pig defects [34]. PCL/ β -TCP composites with different relative compositions and pore sizes were also tested by Lee *et al.* in a beagle model, resulting in good bone formation (new bone volume = $30.50 \pm 16.26 \text{ mm}^3$) and regeneration of the critical mandibular defect [36].

Table 3. Compositions of the composite scaffolds made with PCL and β -TCP.

PCL [% w/w]	β -TCP [% w/w]	Ratio	Printing technology	Composite production	Ref.
50	50	1:1	Ink-jet printer	-	[34]
70	30	2.3:1	Multi-head deposition system	Melting and mixing	[36]
50	50	1:1	3D bioplotter [®] (FDM)	Melting and mixing	[39]
50	50	1:1	FDM	-	[40]
NA ^{a)}	NA ^{a)}	1:2	3D bioplotter [®]	Solvent method	[41]

70	30	2.3:1	Micro extrusion-based 3D printer	Melting and mixing	[47]
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^{a)} NA = not available

Ceramic materials have also been used as fillers for photocurable polymers. For example, Dienel *et al.* reported the development of a cross-linked network of methacrylate poly(trimethylene carbonate) (PTMC) and β -TCP, processed by stereolithography, to fabricate a CSD-specific composite scaffold, for mandibular reconstruction in a minipig model. However, new bone formation was not significantly higher than the one the empty defects (negative control), meaning that the defect size was not critical [64].

In addition to polymer-ceramic, also ceramic-ceramic blends were investigated. One study, for example, combined both HA and β -TCP to obtain a slurry processed with DLP [45]. Unfortunately, after 12 weeks the new bone formation in the experimental group (scaffolds) was lower than the positive control group (Bio-Oss®) [45]. Similarly, Kim *et al.* developed a custom-made HA/ β -TCP scaffold through DLP printing and tested it in beagle dogs with a mandible defect. After 8 weeks of implantation the new bone formation induced by the scaffold was not significantly higher compared to the control group and the non-treated group (empty defect), because, once again, the defect size was not critical [65].

Taken together, mandibular bone regeneration is still facing numerous issues due to the complexity of the native tissue environment to be reconstructed. Composite scaffolds, which combine the properties of polymers and ceramics, are attracting many research efforts. In fact, as reported in **Table 1**, the most used materials are thermoplastic polymers in combination with HA and TCP, as ceramic fillers. These materials are typically processed into scaffolds via extrusion-based deposition systems (**Table 3**). Despite other classes of materials have been investigated, such as photocurable polymers, more *in vivo* studies with CSDs have to be performed to demonstrate the true potential of these materials. Lastly, as proposed by Qin *et al.*, another emerging category of materials in the mandibular BTE field are bioactive glasses and inorganic ions (e.g. Mg^{2+}), which are used to dope scaffolds to enhance osteogenic and angiogenic potential [44]. These materials could be employed alone or in combination with polymers to create innovative composite materials for mandible regeneration.

Additive manufacturing techniques for mandibular regeneration

In mandibular BTE is crucial to reproduce the natural macro-architecture of the tissue to ensure proper functionality and facial physiognomy. It seems evident that a patient-specific approach is of paramount importance. This can be achieved through AM techniques that enable the fabrication of scaffolds layer by layer starting from patient CT scans. Moreover, AM offers the possibility to process various materials, accomplish complex geometries, and generate porous structures. In BTE, a wide variety of techniques are

employed, and they can be categorized in seven classes: material jetting, binder jetting, vat photopolymerization (e.g. DLP, SLA), powder bed fusion (e.g. SLS, selective laser melting (SLM)), material extrusion (e.g. FDM, liquid deposition modeling (LDM), robocasting), and electrospinning [66, 67]. However, for mandibular applications, only SLS, inkjet printing, FDM, LDM, robocasting, and DLP have mostly been employed (**Table 4**).

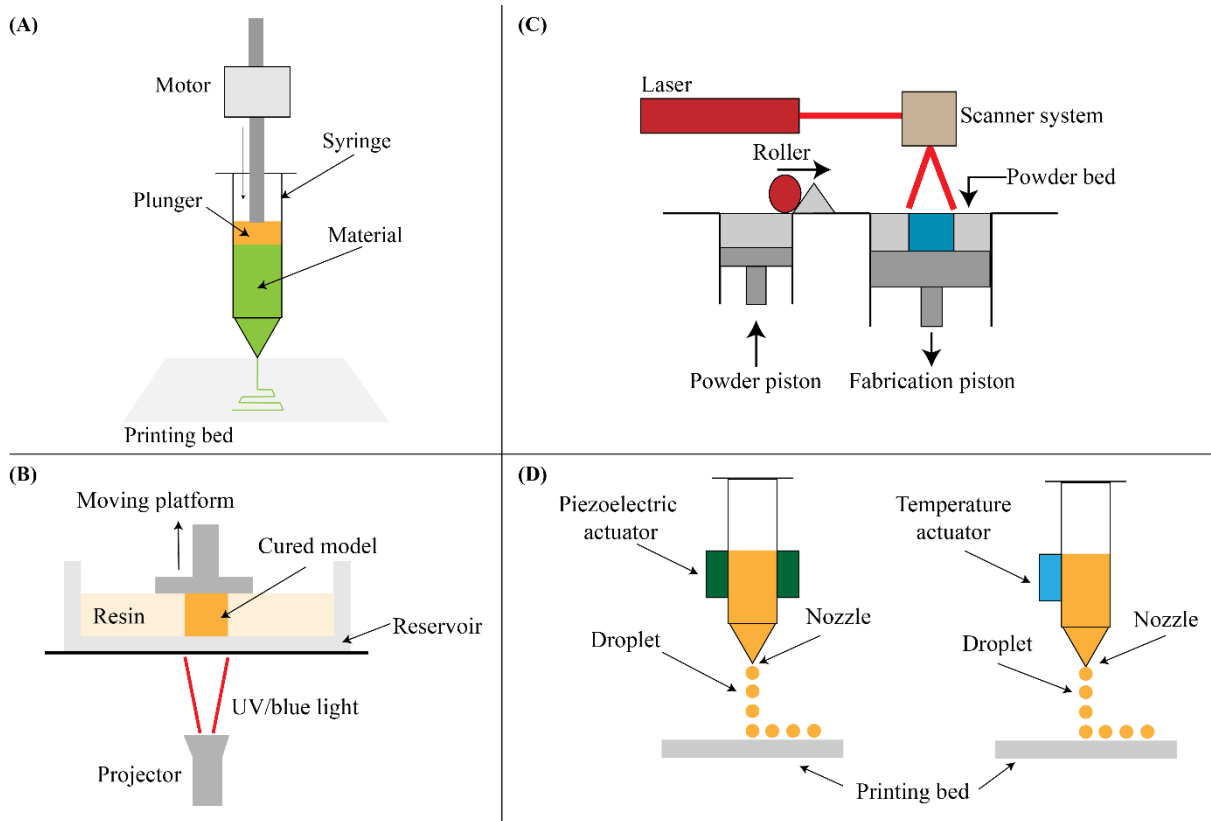


Figure 3. AM techniques used for mandibular BTE. (A) Extrusion-based system; (B) DLP system; (C) SLS process; (D) Inkjet printing process.

LDM, FDM and robocasting are the most used techniques. They are extrusion-based printing systems, in which a single material or a composite is extruded under pressure through a nozzle as a continuous filament (**Figure 3A**) [66]. These techniques are compatible with a wide range of processable materials and have very low production costs. However, they are usually associated with limited resolution ($\sim 300 \mu\text{m}$), and the final parts can exhibit anisotropic behavior [22, 67, 68]. FDM is based on the extrusion of melted materials; those that are mainly used for mandible reconstruction are composites formed by a polymeric (i.e. PCL, PLA, PLGA) and a ceramic part (i.e. TCP, HA). Since the melting process usually implies high temperatures, biological organic components (e.g. growth factors) need to be eventually incorporated at a later time. For instance, Lee *et al.* have successfully printed PCL/ β -TCP composite scaffolds at 120°C , obtaining an interconnected structure with a porosity around 57%. After the printing, the scaffolds were soaked in a collagen solution containing rhBMP-2, to evaluate its effect on bone formation [36].

Similarly, Lopez *et al.* have printed β -TCP scaffolds with robocasting technique, starting from a colloidal ink of the ceramic powders in a water-based solution. This AM technique required the scaffold to be sintered at high temperatures up to 1100°C. After the post-processing procedure, the scaffold was coated with a bioactive molecule of interest (i.e. dipyridamole) [37]. On the contrary, Zhang *et al.* have demonstrated the possibility to print a liquid PCL/ β -TCP composite solution at room temperature, incorporating small molecules during printing [41]. In this way, the molecules have been found in the bulk of the fibers and not just on the surface. Indeed, recently LDM gained attention, because it allows the printing of viscous polymeric or composite solutions at low temperatures [69]. Thus, it could be employed to incorporate biological organic factors (e.g. proteins) in the scaffold structure during the printing without affecting their functionality.

One more AM category used for scaffold fabrication is vat photopolymerization. It involves the use of photosensitive liquid materials, called resins, that can be cured upon exposure to a specific light source, creating a 3D object. Among the vat photopolymerization techniques there are SLA and DLP. SLA exploits a focused laser beam to photopolymerize the material in a specific point; this technique guarantees high levels of detail and surface resolution ($\sim 30\text{-}70\text{ }\mu\text{m}$) [68, 70, 71]. DLP employs instead a blue or UV light from a projector to cure the resin into the desired shape (**Figure 3B**). Even though DLP can obtain objects with lower resolution ($\sim 70\text{-}100\text{ }\mu\text{m}$) than SLA, it benefits of faster processing speed, and extremely good surface quality [66, 68, 72]. For example, Qin *et al.* have fabricated bioceramics scaffolds through DLP technique. Ceramic powders, composed of magnesium-substituted calcium silicates, were mixed with a commercial resin and a photo-initiator. Then, the material have been cured with UV light at a wavelength of 405 nm to create scaffolds with different pore dimensions [44]. Also Ryu *et al.* have fabricated HA/TCP scaffolds through DLP technique. The powders have been mixed with a photo-reactive ceramic resin, photo-initiators, acrylic monomers, and dispersant. The final mixture was printed and cross-linked with UV rays. In this case, the scaffolds required a post-processing (sintering at 1250°C) [45]. Despite the high resolutions that can be reached with vat photopolymerization techniques, there are some challenges that limit their translation into clinical application, such as the limited availability of biocompatible resins and photo-initiators, degradation time of the scaffolds, and high costs [66, 67].

Two other less commonly used AM techniques are SLS and inkjet printing. SLS belongs to the powder bed fusion category, and relies on a laser energy source to locally sinter the material, usually in the form of powder (**Figure 3C**) [23]. Although scaffolds generated with this technique require post-processing operations, SLS allows large and very complex structures to be produced at good resolution ($\sim 500\text{ }\mu\text{m}$) [23, 66-68]. An example is reported in the work of Roskies *et al.* A 3D scaffold of PEEK was manufactured with a CO₂ laser source, obtaining total porosity around 50% and a pore size of 730 μm [49].

Lastly, Konopniki *et al.* have used inkjet printing to fabricate PCL/ β -TCP scaffolds. Inkjet printing consists in the extrusion of droplets of materials through a piezoelectric or thermal actuator (**Figure 3D**) [73]. The scaffold obtained had both macro- and micro-porosity thanks to the drop-by-drop deposition method [34]. To conclude, it is clear that each AM technique has some advantages and disadvantages. However, they are certainly the most promising manufacturing methodology to replicate the mandibular tissue in terms of architecture and composition, in the direction of a more patient-specific treatment.

Table 4. AM techniques and materials used for the fabrication of scaffolds for mandibular regeneration.

AM category	AM technique	Materials	Ref.
Powder bed fusion	SLS	PEEK powder	[49]
Material jetting	Inkjet printing	PCL/ β -TCP composite	[34]
Material extrusion	LDM	HA/PLGA slurry (RT)	[30]
	LDM	PCL/ β -TCP solution + Hydrogel (RT)	[41]
	LDM	PLGA/ β -TCP solution (RT)	[42]
	LDM	PCL/HA solution (RT)	[43]
	LDM	PGLA/HA solution	[51]
	FDM	Melted LayFomm®	[31]
	FDM	Melted lyophilized PLGA/nHA composite	[32]
	FDM	Melted PCL/ β -TCP composite	[33, 35, 36, 39, 40, 47]
	FDM	Melted PLA	[38, 50]
	FDM	Melted β -TCP/stearic acid composite	[46]
	FDM	Melted PCL	[48]
Vat photopolymerization	Robocasting	Colloidal gel formulation of β -TCP	[37, 51]
	DLP	Magnesium-substituted calcium silicate scaffolds (UV)	[44]
	DLP	HA/TCP (UV)	[45]

Scaffold structure and design

In designing a scaffold, it is crucial to take into account the structural and mechanical properties to promote the regeneration of a tissue. From a structural point of view, the bone tissue of the mandible is formed by two outer layers of cortical bone and a thick inner portion of trabecular bone (**Figure 4A**). This structure has been confirmed by bone biopsy samples, harvested from the mandibular symphysis and the ramus [54]. There are evident dissimilarities between mandible's trabeculae and those of other bones (e.g. tibia and ilium). Since different mandibular regions have to fulfil different functions and might present an inhomogeneous dentition state, intra-variations within the mandible bone cancellous structure is also expected [43]. Through μ CT images (**Figure 4B**) it was demonstrated that plate-like trabeculae were present in the symphysis and organized in different directions, creating a complex structure [54]. In the ramus, the plate-like trabeculae were wider and shallower than those found in the symphysis.

In mandibular BTE, the structural properties, in terms of pore shape, pore size and total porosity, influence cell differentiation, angiogenesis and bone growth, and determine to what extent the scaffold conformation resembles the native bone architecture. However, the determination of optimal scaffold pore size for craniomaxillofacial bone repair remains controversial. Several studies report a minimum pore size of 100 μ m to achieve cell migration, and a pore size above 300 μ m to achieve vascularization and bone formation [44]. Moreover, interconnectivity of pores should be 100%, to provide space for cell migration and nutrient transport. This can be easily achieved with AM that allows fine fibre deposition control so to create open-cell porous materials. Lastly, the total porosity should be between 70-80%. In fact, it was demonstrated that higher porosities (65-75%) led to higher *in vivo* bone formation and ALP activity than lower porosities (25%) [20].

Although an increase in porosity and pore size facilitates bone growth, it also affects the structural integrity of the scaffold. If porosity becomes too high, it can negatively influence the mechanical performance of the scaffold. For this reason, the upper limit of total porosity is usually kept around 80%. Therefore, it could be concluded that scaffolds should be fabricated with a pore size in the range of 300-900 μ m and a porosity between 60 and 80%.

Another important morphological parameter is the pore shape, which has been shown to have an impact on bone formation, both *in vitro* and *in vivo* [74, 75]. All the reviewed studies (**Table 1**) have printed scaffolds using a 0-90° fibre orientation, resulting in square-shaped pores, except for two of them, which proposed a different pattern. Interestingly, Lee *et al.* have compared the 0-90° pattern (grid-structure scaffold) with a kagome structure (**Figure 4C**) to improve the low mechanical properties of 3D printed PCL scaffolds, used to fill a calvarial defect [76]. The kagome structure is a quarter-cube honeycomb structure that creates a series of tetrahedra and truncated tetrahedra [77].

The structures were designed to have a porosity of 50%. In mechanical tests, the kagome structure had shown to improve both ultimate compressive stiffness ($UCS = 59.95 \pm 2.91$ MPa) and bending modulus ($E_{flex} = 171.89 \pm 17.75$ MPa), compared to the grid structure ($UCS = 42.73 \pm 5.89$ MPa and $E_{flex} = 73.32 \pm 12.89$ MPa). Similar structures could be translated to mandibular bone application to improve the mechanical properties of the scaffolds. Liu *et al.* have printed three different models, at 90°, 45° and 30° (**Figure 4D**), with a total porosity between 80 and 90%, to compare the different structures in terms of mechanical properties, proliferation and osteogenic differentiation of MC3T3-E1 cells seeded in the scaffolds [43]. The mechanical properties were higher in the 0-90° scaffold than in the other two architectures, with a compressive modulus (E) of 2.0 ± 0.15 MPa for PCL/HA scaffolds. The 0-90° scaffolds showed the highest E, while the 0-30° scaffolds showed the lowest one. Overall, the 0-90° fibre crossing angle structures (FCAS) significantly increased the compressive modulus compared to the 0-30° and 0-45° FCAS in all groups ($p < 0.01$). Furthermore, increased cell proliferation and ALP activity were observed for the 0-90° structure after seven days in the PCL/HA scaffolds *in vitro*.

The last important feature is the mechanical performance of the scaffolds, which should be similar to that of the native tissue in order to promote efficient scaffold osseointegration and support tissue functionality. The cortical bone tissue displays Young's modulus in the range of 15-20 GPa and UCS around 100-200 MPa, whereas the cancellous bone tissue has E in the range of 0.1-2 GPa and UCS around 2-20 MPa [8]. By comparing these physiological values with scaffolds that have been reported so far in literature, it can be assessed that AM scaffolds are not capable to mimic the mechanical integrity of healthy bone tissues. Manjunath *et al.* reported PLA scaffolds with a compressive modulus of 9.98 MPa (SD not available) which was improved to 16.02 MPa (SD not available) with the addition of a PCL matrix [35, 38]. Depending on the structure (e.g. 0-90° pattern, kagome, 0-45° or 0-30° pattern) and the presence of ceramic elements, PCL scaffolds displayed compressive strength values between 10 and 30 MPa and bending modulus between 70 and 170 MPa [36, 39, 40, 76]. These values are at least one order of magnitude lower than cancellous bone and two orders of magnitude lower than cortical bone. In mandibular bone reconstruction applications, mechanical strength and load-bearing capacity are crucial factors, as the mandible experiences high loads during its lifespan.

However, it is worth noting that low mechanical resistance is not necessarily a concern at the onset of bone regeneration, as patients are not allowed to apply loads to the fracture. Moreover, the implants are usually stabilized by metal reconstruction plates. These devices are shaped on the patient's anatomy and can withstand multiple cyclic masticatory loads, as confirmed by mechanical simulations and tests [78, 79]. CAD/CAM methods can be used to fabricate the plates from patient scans, resulting in a more accurate shape and improved mechanical properties [78-80]. For instance, Koper *et al.* have proposed to use topology

optimization, a computational method, to design and fabricate Ti6Al4V SLM plates tailored to the patient's requirements, thereby reducing implant failure [80]. As the bone healing process takes place, new bone forms inside the scaffold, increasing its global stiffness. When the healing is complete, the callus is able to sustain the load independently, and the fixation devices can be safely removed [81].

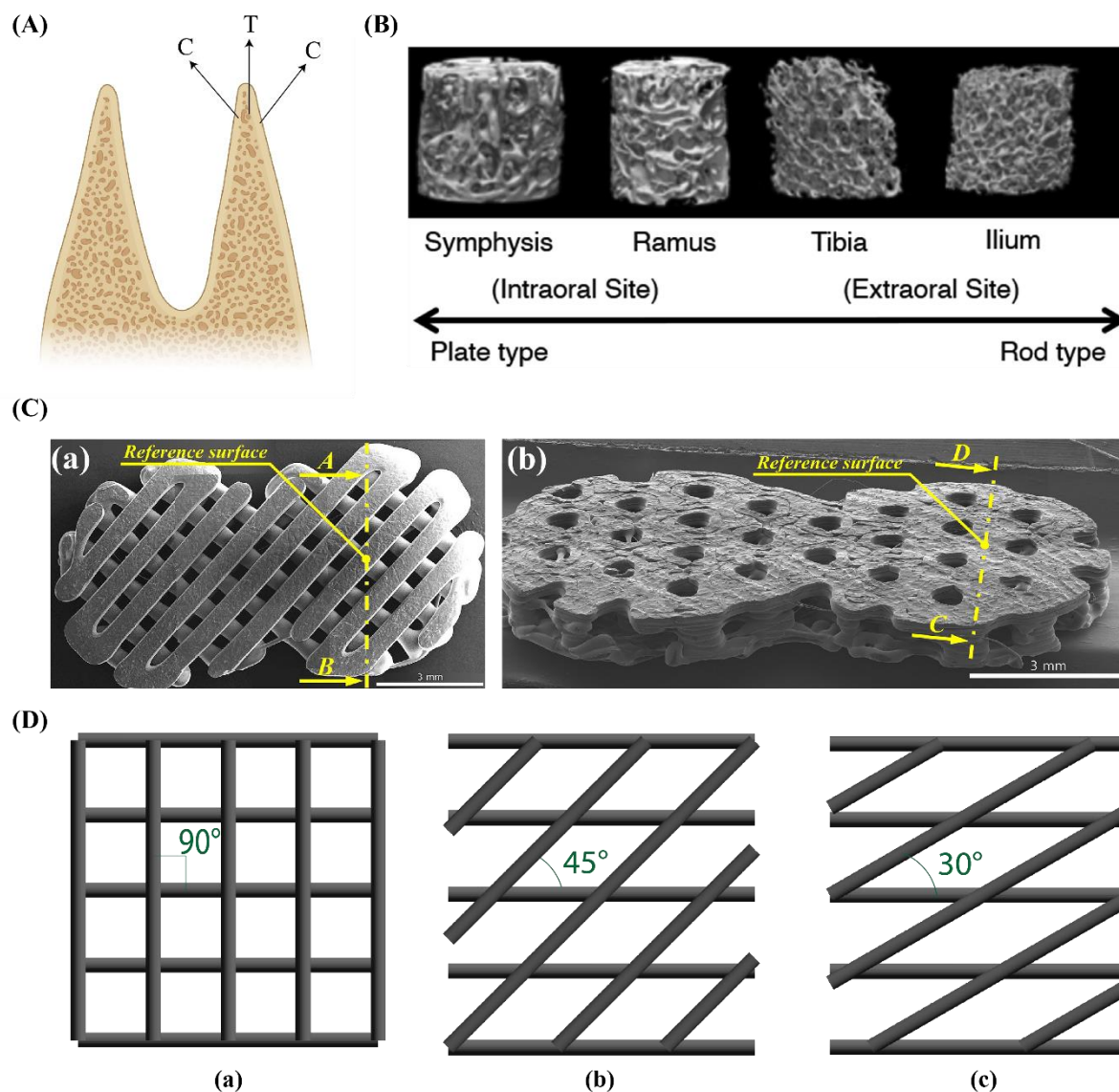


Figure 4. A) Mandibular bone structure: C = cortical bone layer, T = trabecular bone layer. Created with BioRender.com B) Three-dimensional morphological observations of the bone biopsy sample. Reproduced with permission [54]. Copyright 2021, Journal of Oral Biosciences. C) 0-90° pattern (a) and kagome structure (b). Reproduced with permission [76]. Copyright 2019, Material Science and Engineering C. D) Schematics of different fibers orientation: 0-90° (a), 0-45° (b), 0-30° (c).

Bioactive molecules for mandibular regeneration

Additionally, to the importance of selecting the biomaterials and architectures, to engineer a valuable bone construct, also bioactive molecules (e.g. BMP-2) are crucial. In fact, they play a pivotal role in correct tissue formation, repair and homeostasis [82].

However, despite the abundant array of choice, growth factors employed for mandibular BTE are almost exclusively confined to one family: BMPs, and in particular BMP-2.

Mature BMPs are normally secreted from osteoblasts and may either activate their membrane receptors or bind to extracellular matrix proteins, such as collagen, and act as a reservoir for neighbouring cells [83]. These proteins stimulate the differentiation of MSCs into osteoblastic lineage and promote the proliferation of osteoblasts and chondrocytes, being therefore an active player of ossification and bone healing processes [84]. For this reason, a series of studies focused on tailoring scaffolds made of bioactive ceramics and/or polymers with recombinant human BMP-2 (rh-BMP-2). The biomolecule can be directly encapsulated in the bulk structure of the scaffold or first loaded into microcarriers (e.g. hydrogel particles). Moreover, it can be mixed with hydrogels, such as collagen and demineralized bone ECM (bdECM), to be later casted on the scaffold surface [85]. Cao *et al.* obtained very good results with β -TCP scaffolds coated with an rh-BMP-2/gelatin solution and implanted in primates. The BV/TV ratio obtained through μ CT has been reported to increase almost of 20% in the presence of rh-BMP-2 (BV/TV% $\approx$ 85%). Furthermore, cumulative release of rh-BMP-2 from scaffolds has been assessed ($48.5 \pm 6.4\%$ at the 21st day) [42]. Nokhbatolfoghahaei *et al.* have also tested β -TCP scaffolds in several conditions in a canine animal model, and always reported higher histomorphometry values of new bone formation (NB) in the presence of rh-BMP-2 (NB = $48.443 \pm 0.250\%$) [39]. On the other hand, despite reporting a modest trend of bone formation for PCL/ β -TCP scaffolds soaked into a BMP-2/collagen solution (BV/TV $\approx$ 13%), results of Lee *et al.* lacked statistical significance when compared to the control group (PCL/ β -TCP alone) [36]. Similar outcomes were reported in another PCL/ β -TCP scaffold study, where intra-scaffold injections of a rhBMP-2 and bdECM bioink improved bone-to-implant contact ratio (BIC) (BIC = $51.29 \pm 14.64\%$), but new bone formation was again not significantly ameliorated [47]. Lastly, Deng *et al.* work stands out from the other in the context of growth factor delivery due to the employment of ceramics and polymers other than β -TCP and/or PCL. Their PLGA/nanoHA, scaffold complexed with rh-BMP-2-loaded chitosan nanocarriers allowed considerable tissue formation (BV/TV = 33.7% and NB = 45.5%) in rabbits, and efficient growth factor release ($61.38 \pm 2.39\%$ on the 30th day) (**Figure 5B**) [32].

A valid alternative to natural growth factors for stimulating bone formation within a scaffold is the use of small molecules that can modulate osteogenic cellular pathways. β -TCP scaffolds soaked in a dipyridamole solution exhibited excellent bone growth ($26.9 \pm 10\%$) in a rabbit critical-sized mandibular defect (**Figure 5D**). Dipyridamole has been proven, indeed, to indirectly activate adenosine A_{2A} receptors that in turn inhibit osteoclasts differentiation and promote osteoblasts differentiation [37]. Zang *et al.* printed a PCL/TCP/hydrogel mixture filled with two small molecules: resveratrol (RSV) and strontium ranelate (SrRn). RSV and SrRn have several pharmacological effects, lie promoting bone

formation, osteogenic differentiation and the release of angiogenic factors. The combined use of these two molecules significantly raised BV/TV ($\approx 25\%$) *in vivo*, compared to the PCL/TCP scaffolds alone ($\approx 10\%$) (**Figure 5C**). Cumulative release profiles of RSV and SrRn from the 3D printed scaffolds were also reported (SrRn $\approx 70\%$ and RSV $< 30\%$ at day 21st) [41].

BMP-2 and -7 have been approved by the Food and Drug Administration (FDA) for clinical use in open fractures of long bones, non-unions and spinal fusion [86]. However, growth factors have been reported as the cause of some serious dose-related side effects, such as exuberant bone growth, osteolysis and ectopic bone formation [37] and it is difficult to find a consensus in the literature regarding the effective dose of BMP-2 to achieve the expected clinical results [50]. Commercially available products recommended a concentration within the milligram per millilitre range ($1.05\text{--}1.5\text{ mg}\cdot\text{ml}^{-1}$), but this value represents approximately 200'000 times the estimated physiologic concentration of natural BMP-2 found in bone [87]. Moreover, other drawbacks related to BMPs include short half-life, protein instability, control over release rate, and high production costs, which makes routine application not possible yet [41].

From this point of view, small bioactive molecules may represent a valid alternative to growth factors, because they have relatively simple structures, are easy to prepare with consequently lower batch variability and production cost, and are already employed in a wide variety of biomedical applications [37, 41]. Most probably, to limit problematics related to FDA approval and uncontrolled side effects on humans, for future applications, one ideal solution would be the development of synthetic materials with intrinsic osteoinductive capacity [88]. As mentioned in above chapters, differences in chemical composition (calcium phosphate/polymer phase), structural properties (porosity, surface area, grain size and compressive modulus) and dissolution behaviour of a scaffold influence its osteoinductive potential. However, the underlying signal transduction pathways activated by instructive materials are still largely unknown and questions remain to be answered whether this strategy could actually be enough to heal mandibular CSDs and restore bone tissue at physiological level.

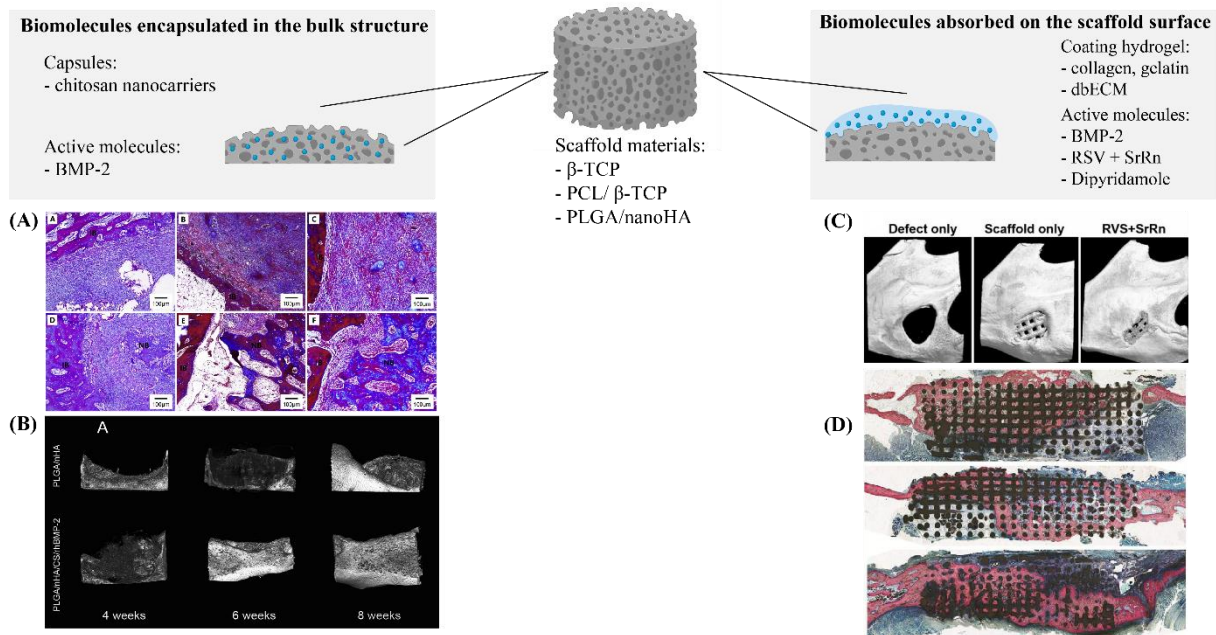


Figure 5. Created with BioRender.com. A) Histological images of the bone defect sites at different time-point. Control group: (A) 4 weeks, (B) 8 weeks, (C) 12 weeks; Experimental group: (D) 4 weeks, (E) 8 weeks, (F) 12 weeks. IB = initial bone, NB = new bone. Reproduced with permission [32]. Copyright 2019, Archives of Oral Biology B) Micro CT reconstruction images of implanted areas at 4, 8, and 12 weeks in the experimental and control groups. Reproduced with permission [32]. Copyright 2019, Archives of Oral Biology. C) Micro CT reconstruction images after 8 weeks in different experimental groups: the empty defect, the defect filled with the scaffold only and the defect filled with the scaffold loaded with the RSV and SrRn. Reproduced with permission [41]. Copyright 2020, Biofabrication. D) Histomorphologic images of the scaffold implanted in the ramus after 8 weeks. Pink = bone, black = residual scaffold, in the control group (above), collagen group (center) and Dipyridamole group (below). Reproduced with permission [37]. Copyright 2019, Plastic and Reconstructive Surgery.

Vascularization strategies for mandibular regeneration

While incredible advances in terms of material properties, scaffold design, and additive manufacturing technologies have been made, the potential promise of craniomaxillofacial BTE have yet to achieve relevant clinical success. AM scaffolds are currently clinically tested for certain applications, such as the treatment of small defects resulting from enucleation of odontogenic cysts or ridge augmentation. For instance, Naik *et al.* conducted a case study in 2019 where PCL scaffolds were implanted in 10 patients with small, non-critical maxilla and mandibular defects. Despite the positive response to the implants, with no pain, swelling or severe inflammation, bone formation within the scaffolds was minimal after 9 months [89]. This result could be attributed to the inert nature of the scaffold material. It is important to notice that this case study, as mentioned above, reported the clinical treatment of a small defect.

In vivo, the maximum distance of a cell from its nearest capillary rarely exceeds 200 μm and is usually less than 100 μm , which is why making a 3D construct with a functional vascular network is considered the most crucial challenge in BTE, especially in critical-size

defects repair [90]. Bone tissue constructs of clinically relevant size (thicker than 1 cm³) [91] and with metabolic activity resembling that one found in the native tissue ($\approx 10^9$ cell cm⁻³) necessitate to be connected to an active blood flow once grafted in a patient, otherwise necrosis will arise faster than blood vessels infiltration into the scaffold (<1 mm day⁻¹) [92]. Furthermore, a proper level of oxygenation is essential for cellular viability, but it is also important for maintaining cellular functions [93]. As a matter of fact, angiogenesis is known to strongly influence osteogenesis. During such processes endothelial cells produce growth factors (e.g. BMP-2, PDGF) that control the recruitment, proliferation, differentiation, function, and/or survival of various cells including osteoblasts and osteoclasts [94].

In the context of mandible reconstruction, only few strategies have been explicitly explored to boost angiogenesis *in vitro* and/or *in vivo* and to promote mass transfer of nutrients and oxygen in the inner core of engineered tissues. A first approach relies on adjusting scaffold key architectural parameters such as porosity, pore size and interconnection [43]. Typically, a pore size ≥ 300 μm is required to facilitate new bone formation and vascularization, while the minimum accepted size seems to be around 100 μm [94]. Qin *et al.* made a comparative analysis of magnesium-substituted calcium silicate scaffolds having the same porosity (58%) but with different pore sizes ($\varnothing$ 480, 600, and 720 μm). Histological analysis of rabbit mandibular defects filled with these scaffolds revealed considerably larger blood vessels in the 600 and 720 μm groups compared to the 480 μm group after 12 weeks from implantation (**Figure 6A**) [44]. The need of macropores for an efficient vascularization *in vivo* was also confirmed in a PVA/PU LayFomm® scaffolds study, where growth of a vascular network was prevented because of lack of porosity in the scaffolds used [31]. Kang *et al.* have reported a novel 3D printing technology (integrated tissue–organ printer (ITOP)) to make multi-material scaffolds with a lattice of microchannels (500×300 μm^2) into human-scale tissue constructs that are permissive to nutrients and oxygen diffusion. With this technique, a scaffold with human mandible critical defect size (3.6 cm $\times$ 3.0 cm $\times$ 1.6 cm) made of PCL/TCP and of a composite hydrogel containing human amniotic fluid-derived stem cells (hAFSCs) was fabricated. After 28 days of culture, osteogenesis was successfully induced *in vitro*. Unfortunately, ITOP potential was not tested *in vivo* specifically for mandibular reconstruction [33].

A second strategy involves the use of a smart scaffold capable of favouring vessel formation by delivering angiogenic factors to cells in culture. As described in the above section, Zhang *et al.* proposed 3D printed PCL/TCP composite scaffolds, with a hydrogel-based bioink, encapsulating bioactive small molecules (RSV and SrRn) [41]. HUVECs treated *in vitro* with the released medium of scaffolds loaded with RSV, showed the highest gene expression levels of VEGFA, platelet endothelial cell adhesion molecule (PECAM)-1, and von Willebrand factor (vWF) (**Figure 6B**).

The most applied scaffold vascularization strategy for mandibular reconstruction consists in exploiting a living bioreactor (i.e. the body) to generate new blood vessels *in vivo*. Such technique involves two steps: a first implantation of the scaffold within a highly vascularized site of the body (e.g. skeletal muscles) and a subsequent graft of the construct at the bone defect site. Microvascular surgery is also required to anastomose the engineered vessels with those at the recipient site [95]. Cao *et al.* shown the superiority of β -TCP scaffolds cultivated in the *latissimus dorsi* muscle of primates over a three-month period [42]. Vascularization of the construct was improved due to penetration of host blood vessels from the neighbouring muscle tissue and, after orthotopic transplantation in a mandibular defect, pronounced effects of bone regeneration were shown (BV/TV $\approx$ 85 % and NB $\approx$ 60 %). Interestingly, PLGA/TCP composite scaffolds prefabricated in the same manner were resorbed too much and could not be used further. Nokhbatolfoghahaei *et al.* also demonstrated a better outcome by using β -TCP scaffolds that preserved a vascularized pedicle from the masseter muscle where they stayed implanted for two months. Significantly greater rate of new bone formation (NB = 48.443 ± 0.250 %) was obtained *in vivo* but, despite all, capillary formation within the constructs could not be proven by histological analysis (**Figure 6C**) [39]. Finally, Konopnicki *et al.* hypothesized that vessels penetration in β -TCP/PCL scaffolds seeded with porcine bone marrow progenitor cells (pBMPCs) was improved by early implanting the constructs in a critical-size mandibular defect (i.e. before the 14th days of *in vitro* culture) [34].

The problem of scaffold vascularization in the context of mandibular BTE remains the narrowest bottleneck that is currently limiting translation of academic research into clinic. Apart from the lack of consistent and well-documented results, there seems also to be a gap in terms of technical evaluation of an efficient vascularized scaffold. The presence of blood vessels is proven almost exclusively by histological images and expression of pro-angiogenic factors. However, it would be suitable to find alternative methodologies able to assess not only the qualitative presence of vessels, but also their functionality, meaning the capability of homogeneously perfusing the engineered construct. Finally, a reliable quantification of the number, structure, hierarchy and distribution of vessels should always be properly evaluated.

Using body's own regenerative potential seems an appealing solution to provide scaffolds with well-formed blood vessels prior implantation in mandibular CSDs. However, this strategy possesses several disadvantages, such as the need of two surgeries, as well as shape and size limitations. In this regard, it would be impossible to regenerate large and articulated bone constructs without excruciating patient discomfort and functional impairment at the ectopic region. A new perspective is therefore of paramount importance. As for the quest of instructive materials to induce bone formation, discovering materials

with an intrinsic angiogenic potential would be desirable also for prompting and guiding vascularization.

A promising route to be better investigated on the mandibular reconstruction topic involves the use of bioactive ions (e.g. Mg, Ce, and Cu) that upon release can lead to increase levels of angiogenic markers [44, 94]. These ions can be exogenously incorporated in the material's matrix or surface, as it has been done for β -TCP, or they can be constitutively present in the material's chemical structure. The latter category includes silicate glasses (i.e. bioactive glasses), which upon dissolution can release Si, Ca, P, and Na ions that were proven to stimulate osteogenesis, neovascularization and/or angiogenesis, and enzymatic activity [96, 97]. With such instructive materials, not only the laborious procedure of the living bioreactor strategy can be avoided, but many drawbacks related to angiogenic growth factors' approval from FDA could be bypassed.

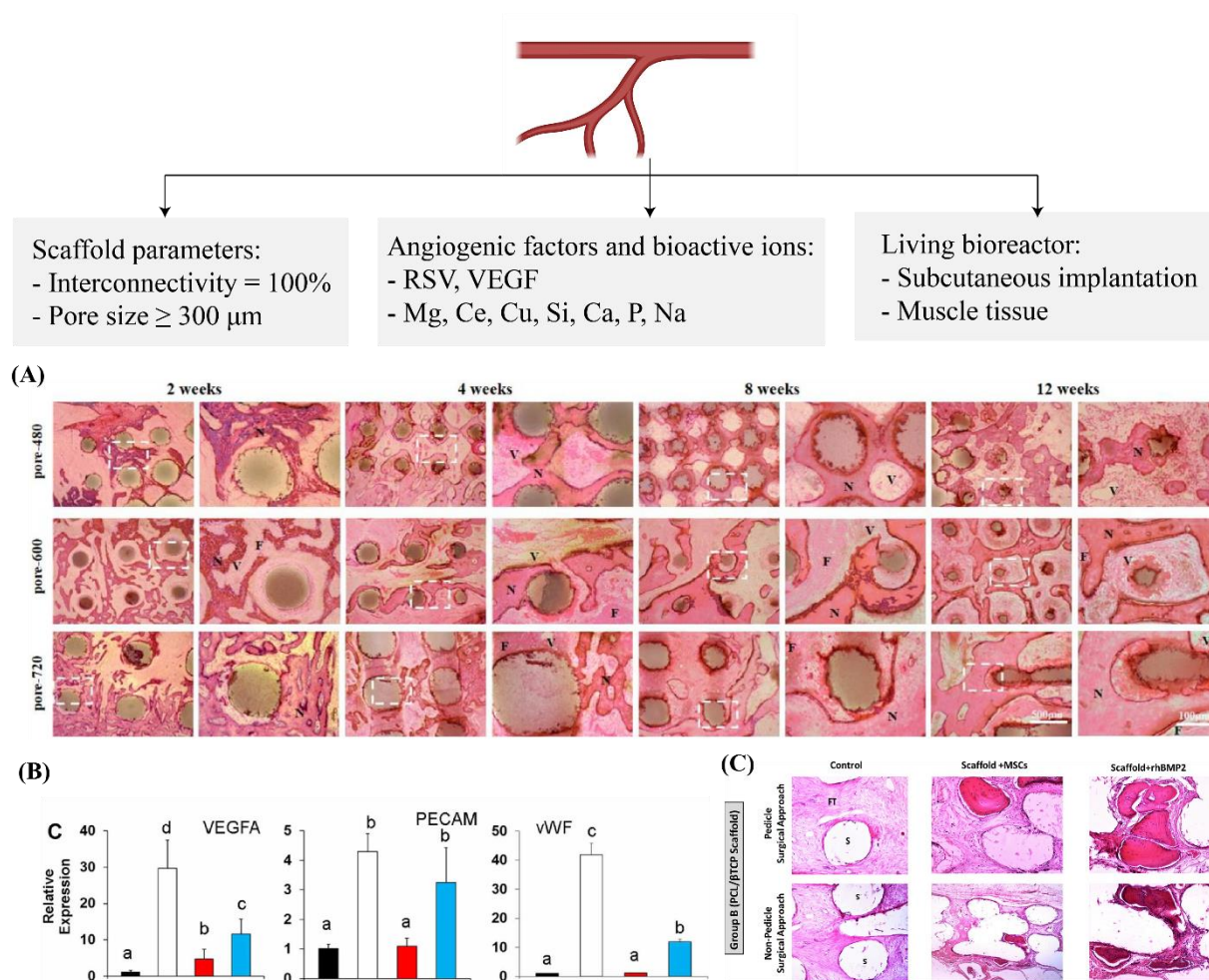


Figure 6. Created with BioRender.com. A) Histological analysis of H/E staining from 2 and 12 weeks, N = new bone, F = fiber, V = vessels. Reproduced and adapted with permission [44]. Copyright 2022, Journal of Tissue Engineering and Regenerative Medicine. B) qPCR analysis of VEGFA, PECAM and vWF genes for HUVEC cells. Bars that have different letters are statistically different from each other. Reproduced and adapted with permission [41]. Copyright 2020, Biofabrication. C) Histological images of H/E-stained sections from mandibular defects at 12 weeks post-surgery. Reproduced and adapted with permission [39]. Copyright 2022, Journal of Biomedical Materials Research.

As a final remark, in human bones, blood supply is accompanied by innervation that also plays a crucial role in maintain tissue homeostasis. The maxillofacial region is highly innervated. The mandible is indeed crossed by the inferior alveolar nerve responsible of sensory functions of the tissue [98]. When a patient undergoes a mandibular resection surgery part of this nerve is removed, causing a loss of sensation in the lip and chin that can result in impaired functions (e.g. chewing, and speaking). Despite the known importance, innervation seems not to be taken into consideration in BTE approaches for mandibular regeneration. In fact, none of the reviewed studies (**Table 1**) mentioned an approach for scaffold innervation. Recently, it was demonstrated that the nerve growth factor (NGF) can enhance bone regeneration by improving the repair of sensory nerves in the mandible [99]. Moreover, NGF can enhance the activity of osteocytes, promote the differentiation and mineralization of osteoblasts, and vascularization, during the process of implant-bone binding [100]. Ye *et al.* proposed a biomimetic coating made of NGF-chondroitin sulphate (CS)-hydroxyapatite (HA) to coat titanium implants, ameliorating early osseointegration and nerve regeneration in the mandible of beagle dogs [100]. Therefore, since osseointegration, vascularization and innervation of the implants are strongly interconnected, more efforts should be placed in this direction in future studies.

Cell therapy

Cell therapy is a common approach when trying to repair critical-sized bone defects. For many years, researchers have been exploring the possibility of using different types of stem cells in combination with scaffolds for BTE. The classical tissue engineering paradigm foresees five critical steps: harvesting cells from the patient (i); expanding cells *in vitro* (ii); seeding cells onto a scaffold (iii); maturation of cells with the potential help of suitable growth factors and a bioreactor system (iv); implantation of the functional biological construct into the patient. (**Figure 7A**). The most appealing cell source are MSCs. These cells have the ability to differentiate into several cell types, including the osteoblastic lineage, and can be harvested from autologous sites (e.g. bone marrow, adipose tissue) so to limit immune response when implanted into the patient.

Among the studies revised, only six followed the traditional tissue engineering approach of seeding cells on scaffolds before the implantation into the animal model. Konopnicki *et al.* have seeded pBMPCs on PCL/ β -TCP scaffolds and the constructs were incubated for 14 days in a rotational oxygen-permeable bioreactor. After, they were implanted in the defects and compared to unseeded scaffolds and to empty defects. Interestingly, a higher value of percentage surface area (PSA) was shown for the seeded scaffolds, reaching a value of 22.1 ± 22.45 %, compared to the unseeded scaffolds ($PSA = 1.87 \pm 3.66$ %) [34]. Lee *et al.* treated the defects with PCL/ β -TCP scaffolds seeded with ADSCs aggregates (around 350 Hu after 8 weeks, compared to the 250 Hu of the scaffold alone) [35]. Also in this

case, the results suggested that the use of cells seeded on the scaffolds could enhance the mandibular reconstruction. A similar improvement was obtained by Nokhbatolfoghahaei *et al.* and Park *et al.*, who seeded MSCs and TMSCs on PCL/ β -TCP scaffolds, showing quantitatively the improvement in bone regeneration ($15.430\% \pm 0.547\%$ and $5.813\% \pm 1.345\%$), compared to scaffolds alone or scaffolds combined only with growth factors [39, 40]. Finally, Temple *et al.* and Roskies *et al.* have proven the efficacy of the seeding of ACSCs aggregates on both PCL (**Figure 7C**) and PEKK (**Figure 7D**) scaffolds, showing a higher cell infiltration in the seeded scaffolds after 7 days of *in vivo* implantation [48, 49].

An alternative direction to the traditional tissue engineering paradigm is based on the implantation of a cell-free scaffold to induce tissue growth directly *in vivo*. As a matter of fact, culturing cells on scaffolds necessitate the help of advanced equipment (e.g. bioreactors, hoods, incubators) and specialised staff. In addition, since stem cell proliferative and differentiation potential is highly dependent on patient age, results may vary or even become ineffective in elderly. As described in above sections, it would be ideal to stimulate bone formation only through material intrinsic properties and to exploit the host body regenerative potential.

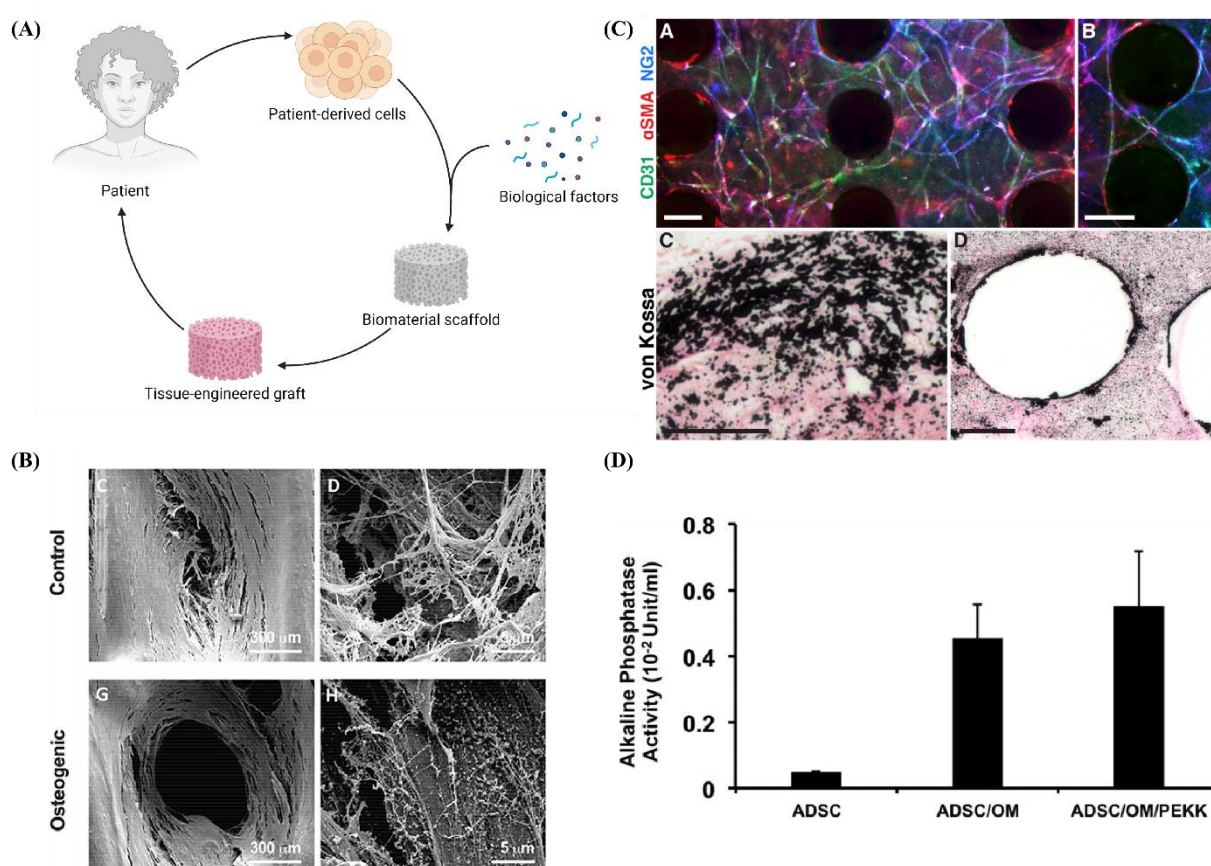


Figure 7. A) Traditional cell therapy approach. Created with BioRender.com. B) SEM images of the seeded scaffolds after 21 days of culture, showing the cells and the matrix filling the pores of the

scaffold. Reproduced under the terms of the CC-BY 4.0 license [31]. Copyright 2020, Copyright 2020, Cooke, Ramirez-GarciaLuna, Rangel-Berridi, Park, Nazhat, Weber, Henderson and Rosenzweig. C) In vitro vascularization and mineralization of ASC aggregates seeded on PCL scaffolds and cultured for 14 days in vascular (A-B) or osteogenic medium (C-D). Reproduced and adapted with permission [48]. Copyright 2014, Journal of Biomedical Materials Research. D) ALP activity of ADSC aggregates alone, in osteogenic medium (OM) and of aggregates seeded on scaffolds and cultured in culture medium (OM/PEKK). Reproduced and adapted with permission [49]. Copyright 2017, Laryngoscope.

***In vivo* studies**

Different strategies to improve bone regeneration *in vivo* have been explored, including the use of cell sources, growth factors, small molecules and prevascularization. However, since experiments have been performed on different animal models, and most importantly, on mandible CSDs of different size, data comparison is not straightforward.

The inhomogeneity of defect size can be attributed to the absence of a clear and quantitative definition of CSD, which has not yet been postulated. This lack of clarity has led to misinterpretation of data and misleading evaluation of tissue engineering approaches. While certain studies have defined a CSD as a segmental defect of a length of 2-2.5 times the diameter of the treated bone, there is still a need for a precise and standardized definition [101]. The CSD dimension depends on the animal model, which can be small or large, and on the site of implantation. Small animals include rats, rabbits, mice and guinea pigs, and they are usually used for first steps of *in vivo* experiments, because of their accessibility, relatively low cost and ethical acceptance [102]. Large animal models, on the contrary, are more clinically relevant human models, and they include pigs, dogs, primates and goats/sheep [102]. In **Table 5**, some studies are reported as examples of different size of calvaria defects, varying the animal model.

Table 5. Examples of CSD dimensions for different animal models and implantation sites.

Animal model	Implantation site	CSD dimension	Ref.
Rabbits	Calvarial	10 mm diameter x 1.2 mm height	[103]
Rodents	Calvarial	4 mm diameter x 2 mm height 8 mm diameter x 0.8 mm height	[104, 105]
Pigs	Calvarial	10 mm diameter x 10 mm height	[106]
Sheep/goats	Tibial*	20 mm length x 21 mm diameter	[107]

*In general, this animal model is exclusively used for long bone defects.

In this section, we discuss CSD dimensions and volumes in relation to reported values of BV/TV. A parameter called BV/TV $_{\Delta}$ has been calculated as the difference between the

BV/TV of the experimental (BV/TV_{exp}) and the control (BV/TV_{ctrl}) group to obtain a value of bone formation comparable among different studies.

Ten studies (**Table 6**) adopted a small animal model to test the efficacy of their scaffolds. In general, it is possible to state that defects' volume of small size animal models was between 6.2 and 750 mm³ with smallest linear dimension in the millimetre range (0.5-5 mm), whereas BV/TV_{Δ} spanned from 5 to 15.93% when the CSD of the control group was filled with some material (usually a scaffold without biomolecules nor cells). Finally, it is worth noticing that the duration of the studies is heterogeneous but always in the order of few weeks (usually 6, 8 or 12). In their work, Cooke *et al.* have shown that $BV/TV\%$ was enhanced by $15.93 \pm 17.40\%$ in defects filled with LayFomm[®] scaffolds when compared with the commercial Norian CRS Putty [31]. In addition, Zhang *et al.* have demonstrated a similar increase of bone formation ($BV/TV_{\Delta} = 14\%$) in PCL/ β -TCP scaffolds loaded with RSV and SrRn with respect to PCL/ β -TCP scaffolds alone [41]. Even though such values of bone formation are among the highest reported in small size animal model studies, it must be pointed out that the overall scaffold volumes were just 30 mm³ (smallest dimension 2 mm) and 9.6 mm³ (smallest dimension 1 mm), respectively. In terms of construct viability contained dimensions is an advantage since oxygen and nutrients diffusional transport to inner cells will be more easily granted. With this in mind, Park *et al.* and Lopez *et al.* (**Figure 8A**) results are therefore more significant, since the authors have still reached comparable BV/TV_{Δ} values (14.32% and 14.60%) but with scaffolds of bigger volumes (400 mm³ and 300 mm³) [37, 40]. The only study comparing an experimental group (PEEK scaffold seeded with ADSCs) with an empty defect as control group registered, as expected, a quite high BV/TV_{Δ} value (61.27%), being BV/TV_{ctrl} equal to zero [49].

Table 6. Small animal model studies. Defects' dimensions and volume correlated with BV/TV_{Δ} .

Animal	Dimensions [mm]	Volume [mm ³]	BV/TV_{exp} (%)	BV/TV_{ctrl} (%)	BV/TV_{Δ} (%)	Time (weeks)	Ref
Rat	$\varnothing_{out}=5$, $\varnothing_{in}=1.2$, $h=2.5$	46.26	25 *	18*	7	8	[30]
Rat	5x2x3	30	30.26 ± 9.46	14.33 ± 7.94	15.93 ± 17.40	6	[31]
Rabbit	13x6x4	312	33.7	24 *	9.7	12	[32]
Rabbit	10x10x3	300	26.9 ± 10.7	12.3 ± 8.3	14.60 ± 19	8	[37]
Rabbit	10x8x5	400	53.10; 57.44	43.12	9.98; 14.32	12	[40]
Rat	$\varnothing=3.5$, $h=1$	9.6	24 *	10*	14	8	[41]

Rabbit	10x6x4	240	23 *	18 *; 14 *	5; 9	12	[44]
Rat	4x4x2	32	NA	NA	-	1	[48]
Rabbit	15x10x5	750	61.27 ± 8.24	0	61.27 ± 8.24	20	[49]
Rat	Ø=4 mm, h=0.5	6.2	17.8 ± 3.77	13.58 ± 6.08	4.22 ± 8.85	4	[51]

* The exact value was not stated in the paper. NA: data not available

After all, the ultimate goal of the use of an animal model is to transfer the obtained results to clinical practice. Small size animals' drawbacks include limited or rapid cortical remodelling and secondary osteon formation, thinner femoral condyle cartilage and a cortical bone composition (e.g., hydroxyproline and protein content) differing from that of humans. That is why large animal models (e.g. sheep, goats, pigs, and dogs) are required for demonstrating sufficient translational capacity.

Defect volumes of large size animal model studies under review were in the order of few thousands of mm³, with the lowest value of 400 mm³ and the highest of 12000 mm³ (**Table 7**). Interestingly, the smallest scaffold linear dimension was still in the millimetre range (5-10 mm), just doubling that one of small size animals. This asymmetric conformation still favours mass transport of oxygen by diffusion inside the scaffolds, despite the increment in construct volume of nearly two orders of magnitude. Unfortunately, BV/TV% values were calculated by very few groups. Cao *et al.* have reported a value of BV/TV_Δ of nearly 60% for β-TCP scaffolds loaded with rhBMP-2, which was further increased to 80% when such scaffold was prefabricated over three months to increase vascularization (**Figure 8B**) [42]. Considering the high BV/TV_Δ and the large defect size (volume = 3000 mm³) this tissue engineering approach can be considered the best among the articles reviewed. On the other hand, Bouyer *et al.* used a clinical-grade PLA coated with a polyelectrolyte film loaded with BMP-2 at 110 µg·cm⁻³ (BMP110) and 50 µg·cm⁻³ (BMP50) to fill the biggest defect reported (volume = 12000 mm³; smallest dimension = 10 mm) [50]. Despite the BMP110 group induced an increase in bone volume compared to the control group (autologous bone grafts), the difference (BV/TV_Δ = 18.33%) was found not to be significant.

Table 7. Large animal model studies. Defects' dimensions and volume correlated with BV/TV_Δ.

Animal	Dimensions [mm]	Volume [mm³]	BV/TV_{exp} (%)	BV/TV_{ctrl} (%)	BV/TV_Δ (%)	Time (weeks)	Ref
Mini Pig	20x20x7	2800	NA	NA	-	8	[34]
Dog	NA	-	NA	NA	-	8	[35]

Dog	20x10x10	2000	13*; 8*	4*	9; 4	12	[36]
Dog	25x10x8	2000	NA	NA	-	12	[39]
Primate	20x15x10	3000	65*;85*	5*	60; 80	12	[42]
Dog	9x9x10	810	43.79 ± 19.35	45.49 ± 12.09	-1.70 ± 31.44	12	[45]
Mini pig	30x24x5	3600	NA	NA	-	6	[46]
Dog	NA	NA	NA	NA	-	12	[47]
Mini pig	40x30x10	12000	63.33 [£] ; 40 [£]	45 [£]	18.33; -5	13	[50]
Goat	40x15x10	6000	NA	NA	-	24	[53]

* The exact value was not stated in the paper. [£] calculated by values of BV and TV reported in the paper. NA: data not available.

The presented results suggest that scaffolds implanted with the addition of cells and/or growth factors performed better in terms of bone formation compared to scaffolds implanted alone, regardless of the animal model and regeneration time. Therefore, although as highlighted above, the use of biological components should ideally be avoided, being an additional element that could delay the approval and commercialization of a scaffold, to date it still appears to be an indispensable component of any tissue engineering strategy.

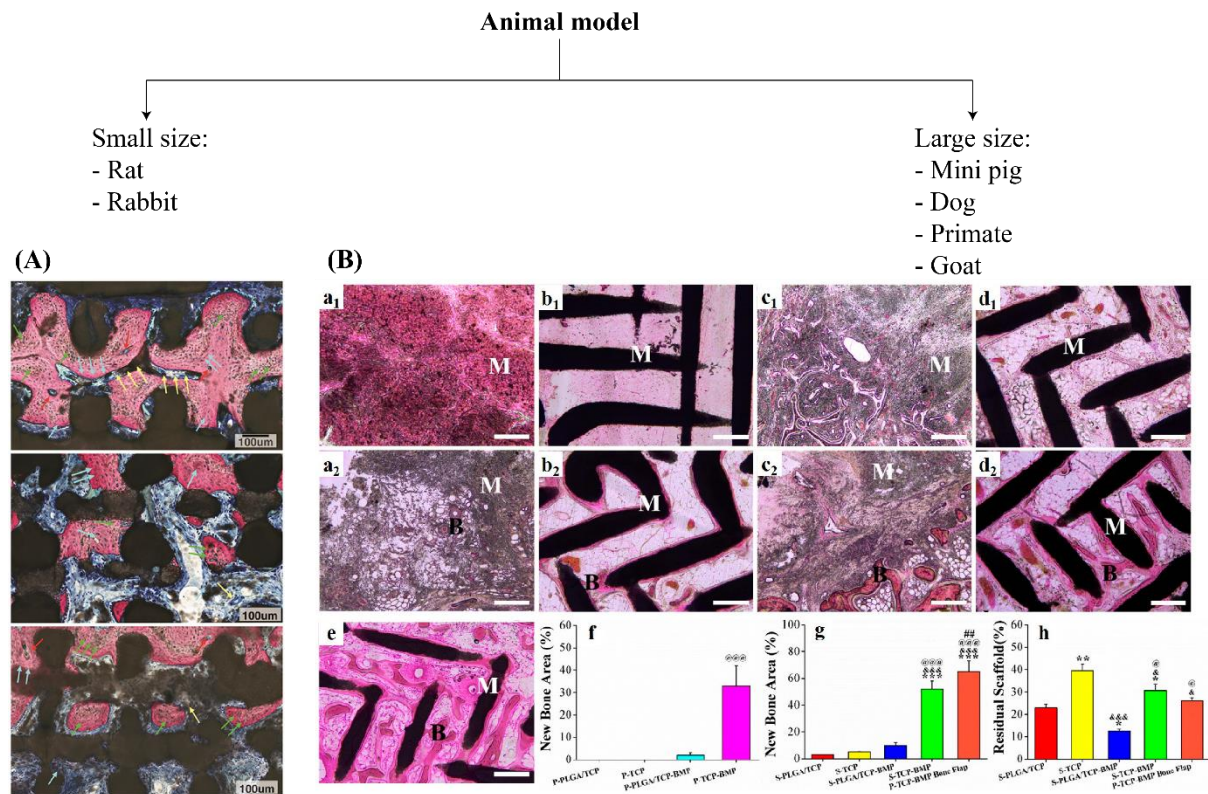


Figure 8. A) Histological evaluation of bone remodelling in Dipyridamole group (Above), Collagen group (Center) and Control group (Below). Vascular structure is depicted as well (yellow arrow). Reproduced with permission [37]. Copyright 2019, Plastic and Reconstructive Surgery. B) H&E staining after 12 weeks of implantation with P-PLGA/TCP(a), P-TCP (b), S-PLGA/TCP (c), S-TCP (d), and P-TCP-BMP (e) scaffolds, without or with rhBMP-2 coating. M= materials, B = bone. Percentage of new bone in histological sections obtained from ectopically (f) and orthotopically (g) implanted samples. Percentage of non-degraded scaffolds (h). Scale bar = 400 µm. Reproduced under the terms of the CC-BY 4.0 license [42]. Copyright 2021, American Chemical Society.

Conclusions, challenges and future perspectives

The mandibular bone tissue presents unique features in terms of origin, function, and composition, when compared to other long bones of the human body. Therefore, its regeneration through BTE approaches has to address different requirements. While there are several synthetic substitutes available on the market for small maxillofacial defects, the gold standard for CSDs treatment remains limited to the fibula flap autografts.

AM is a promising technique to fabricate novel implants for CSDs that may serve as alternative treatments. Although multiple AM techniques have emerged, extrusion-based systems are mostly employed for mandibular BTE applications. It allows creating porous scaffolds, in an easy, accessible, and low-cost way, and with complex geometries, mimicking the micro-architecture of the mandible. Moreover, a wide range of biomaterials or composites can be processed to approach the native mandible composition, ensuring the osteoinductive and -conductive properties. Specifically, for mandibular BTE, the most used materials are composites made of β -TCP and PCL. Although scaffolds mechanics can

be tuned by composite selection or geometry, current scaffolds are not able to withstand the high mechanical loading of the jaw. To ensure this stability in the early healing phase, metal reconstruction plates have to be co-implanted. AM scaffolds can be integrated with growth factors and biomolecules (both natural and synthetic ones) to enhance osteo- and angiogenic differentiation of resident stem cells. To stimulate osteoblastic differentiation, BMP-2 is most commonly used as biomolecule. However, the use of BMPs is associated with dose-related side effects, pushing research towards controlled release strategies, such as the use of hydrogels, carriers, or coatings, integrated in the scaffolds. Moreover, scaffolds can be pre-seeded with cells. The most commonly used cellular components are MSCs, thanks to their potential to differentiate into osteoblasts. They can be harvested from the patient itself, to use them as an autologous component in the synthetic scaffolds.

In the review, we specifically investigated the effect of scaffolds on *in vivo* new bone formation, comparing different CSD dimensions and animal models. The results suggested that the combination of scaffold materials, growth factor and/or pre-seeded cells is the most effective approach in terms of bone formation.

Regardless, nowadays, significant challenges are limiting the translations of the constructs into clinics. Firstly, the main limitation is the lack of vascularization within the scaffolds, which induces necrosis due to absence of nutrients and oxygen. Here, the best approach seems to be the pre-vascularization of the constructs. However, this practice is time demanding and surgically complex, requiring the connection between the newly formed vessels and the natural ones. Secondly, as previously mentioned, the innervation of the constructs is an aspect that has not yet been investigated. Therefore, in this area more extensive research is required. Lastly, this review has shown the lack of a quantitative and precise definition of what is to be considered a CSD, resulting in inhomogeneity in the methodological approaches in the testing of scaffolds *in vivo*, and in difficult and misleading evaluation of the different tissue engineering approaches. Therefore, it would be recommended to establish standard guidelines, with a defined size for various animal models and defect sites. In conclusion, although research on AM scaffolds, integrated with bioactive molecules and cell therapy, has made many advances in the last decades, there is still room for improvement in next-generation scaffolds.

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CHAPTER 3

PCL/ β -TCP composite scaffolds with advanced geometries promote hMSCs osteogenic differentiation

Sophia Dalfino, Elena Olaret, Marco Piazzoni, Paolo Savadori, Izabela Stancu, Gianluca Tartaglia, Claudia Dolci, Lorenzo Moroni

Dalfino et al., *Tissue Engineering Part A*, 2025, 10.1089/ten.TEA.2024.0030

Abstract

Critical-sized mandibular bone defects, arising from e.g. resections after tumor surgeries, are currently treated with autogenous bone grafts. This treatment is considered very invasive and is associated with limitations such as morbidity, and graft resorption. Tissue engineering approaches propose to use 3D scaffolds that combine structural features, biomaterial properties, cells and biomolecules to create biomimetic constructs. However, mimicking the complex anatomy and composition of the mandible poses a challenge in scaffold design. In our study, we evaluated the dual effect of complex pore geometry and material composition on the osteogenic potential of 3D printed scaffolds. The scaffolds were made of polycaprolactone (PCL) alone (TCP0), or with a high concentration of β -tricalcium phosphate (β -TCP) up to 40% w/w (TCP40), with two complex pore geometries, namely a star- (S) and a diamond-like (D) shape. SEM and micro-CT images confirmed high fidelity during the printing process. The D-scaffolds displayed higher compressive moduli than the corresponding S-scaffolds. TCP40 scaffolds in simulated body fluid showed deposition of minerals on the surface after 28 days. Subsequently, we assessed the differentiation of seeded bone marrow-derived human mesenchymal stromal cells (hMSCs) over 28 days. The early expression of RUNX2 in the cell nuclei confirmed the commitment towards an osteogenic phenotype. Moreover, ALP activity and collagen deposition displayed an increasing trend in the D-scaffolds. Collagen type I was mainly present in the deposited ECM, confirming deposition of bone matrix. Lastly, Alizarin Red staining showed successful mineralization on all the TCP40 samples, with higher values for the S-shaped scaffolds. Taken together, our study demonstrated that the complex pore architectures of scaffolds comprised TCP40 stimulated osteogenic differentiation and mineralization of hMSCs *in vitro*. Future research will aim to validate these findings *in vivo*.

Keywords

Bone tissue engineering, Additive manufacturing, Composite scaffolds, Pore shape, hMSC differentiation

Introduction

Mandibular critical-sized defects, resulting from traumatic injuries or tumor resections, fail to spontaneously heal, requiring surgical interventions to be repaired [1, 2]. Nowadays, the golden standard treatment is implanting an autologous bone graft, harvested from the fibula of the patient [3]. However, this very invasive procedure is associated with multiple drawbacks such as donor site morbidity, graft resorption, and risk of infections [4-6]. As an alternative solution, tissue engineering principles have been used to design mandibular implants (i.e. scaffolds), combining cells, biomaterials, and biomolecules [7]. To achieve successful regeneration, features of the complex mandibular anatomy and composition must be integrated in the constructs. To this end, additive manufacturing techniques enable the fabrication of scaffolds with high control over the design, and in a patient-specific manner [8]. Among these biofabrication technologies, melt-extrusion based manufacturing technology has been most frequently used as they permit deposition of biomaterials, resembling the native tissue composition, into a porous scaffold with intricate geometry [9].

Melt-extrusion based techniques typically use synthetic thermoplastic materials [10]. For instance, medical grade polycaprolactone (PCL) has previously been selected for scaffold fabrication for bone applications, as it is biocompatible, biodegradable in the long term, and offer good mechanical properties [11-15]. However, PCL is known to be inert [16]. Therefore, its bioactivity can be enhanced in combination with ceramic materials, such as tricalcium phosphates (TCP) [11, 16-18]. Simultaneously, the incorporation of ceramics leads to biomimicry of the natural bone matrix, which contains an inorganic phase of hydroxyapatite minerals embedded in a collagen-based organic phase [19]. Moreover, the incorporation of ceramic particles reinforces the polymeric matrix, enhancing the mechanical performance of the final scaffolds while solving the intrinsic brittleness of scaffolds made of ceramic only [20, 21]. Among different ceramics, TCP has higher solubility and faster resorption rate compared for example to hydroxyapatite. Therefore, it can lead to a rapid bone formation after implantation, improving tissue regeneration [7]. Different studies reported the successful use of PCL-TCP composite scaffolds to enhance the osteogenic differentiation of seeded adult stem cells and *in vivo* bone regeneration [19, 22-24]. For example, Khojasteh et al. fabricated PCL scaffolds containing 20% w/w TCP to evaluate their effect on healing of vertical bone critical sized defects in a dog's mandible model [22]. Another study used PCL with 30% w/w TCP, to promote *in vitro* osteogenic differentiation of bone marrow stem cells [25].

In addition to the composition of the scaffolds, studies have also reported that pores geometry affects osteogenesis. For example, Werner et al. demonstrated that 3D substrate curvatures can modulate the cytoskeletal forces acting on the nucleus, driving cell differentiation towards an osteogenic phenotype [26]. Di Luca et al. showed that

rhomboidal pore shapes promoted better osteogenic differentiation of human mesenchymal stromal cells (hMSCs), when compared to typical woodpile constructs [27]. Recently, mathematical models describing triply periodic minimal surfaces (TPMS) were also applied to scaffold design. Minimal surfaces are found in nature, thus they can be useful to achieve biomimicry of the scaffolds [28]. For instance, the geometries described by the TPMS models resemble the native trabecular bone, characterized by curved surfaces [29]. Among the different TPMS structures, the diamond was shown to be promising in terms of cell migration and (osteogenic) differentiation, because of greater curvatures [30, 31].

In this study, we aimed to assess the combined effect of the pore geometry and material composition on the osteogenic potential of 3D printed scaffolds. We used a composite material made of a medical grade PCL matrix, containing a high content of β -TCP (40% w/w). In fact, to the best of our knowledge, studies that used melt-extrusion techniques for mandibular applications reported a maximum incorporation of 30% w/w of TCP content [22, 25, 32]. Given that the inorganic component of bone typically constitutes about 60% of the tissue [33], incorporating such a high amount of TCP in our composite represents a step forward mimicking the natural mineral bone composition.

In addition, instead of a typical woodpile architecture, the scaffolds were fabricated with two advanced pore geometries, a diamond- (D) and a star- (S) shape. First, the morphology of the scaffolds was optimized and evaluated with SEM and micro-CT. Then, the scaffolds were tested *in vitro* for mechanical performance and bioactivity. Finally, the potential of the scaffolds to induce osteogenic differentiation of hMSCs was investigated during a 28-day culture period *in vitro*. We assessed the expression of osteogenic markers, such as RUNX2, ALP activity, collagen deposition, and mineralization of the matrix, from the gene to the protein level. Our results pave the way for a novel approach in scaffold design for mandibular critical-sized defects.

Materials and methods Medical grade PCL (PURASORB® PC12, Mw 72 kDa) was provided by Corbion (Amsterdam, The Netherlands). β -TCP powder was provided by Kuros (The Netherlands). All chemicals were bought from Sigma Aldrich, unless stated otherwise.

Composite preparation and characterization

β -TCP preparation and characterization

The β -TCP particle size was decreased with ball milling, performed in MilliQ water for 72h. The particle size was determined with dynamic light scattering (DLS, Malvern Zetasizer Nano ZS, Malvern Instruments Ltd., UK). The measurement was done at 25°C, using 0.1% w/v of particles in distilled water (dH₂O), in a polystyrene disposable cuvette.

In addition, the milled particles were gold coated and imaged with scanning electron microscopy (SEM, Jeol JSM-IT200 InTouchScope scanning electron microscope) at a working distance of 10 mm using a voltage of 10 keV.

Composite preparation

The composite material (TCP40) of PC12 and TCP (60:40 w/w) was prepared via a blending strategy, as depicted in the schematic in **Figure 1A**. First, the polymer was dissolved in tetrahydrofuran at $T = 30\text{ }^{\circ}\text{C}$ under stirring. Then, the TCP powder was added, and the mixture was stirred until homogeneous dispersion of the powder. The solution was mixed with a mechanical stirrer to reach a homogeneous dispersion. Finally, the particle mixture was poured in absolute ethanol (1:4 v/v) to obtain the final composite paste. The final paste was then let dry in a vacuum oven overnight at $T = 30\text{ }^{\circ}\text{C}$.

Thermogravimetric analysis

The efficiency of the blending method was evaluated with a thermogravimetric analysis (TGA, TA Instruments TGA550). Briefly, PCL (TCP0) and TCP40 were heated to $700\text{ }^{\circ}\text{C}$ at a ramp of temperature of $10^{\circ}\text{C}\cdot\text{min}^{-1}$. Then, the temperature was kept constant for 5 min, before it was decreased at the same rate back to $25\text{ }^{\circ}\text{C}$. The final weight percentage indicated the percentage of TCP in the composite.

Chemical characterization

The chemical composition of TCP0 and TCP40 was investigated with Fourier-transform-infrared (FTIR) spectroscopy (Nicolet iS50, Thermo Scientific) and X-ray diffraction (XRD, D2 phaser, Bruker, USA). FTIR was performed in the range $4000\text{--}400\text{ cm}^{-1}$, running 32 scans. XRD was conducted in the range of $7^{\circ} \leq 2\theta \leq 60^{\circ}$, with increments of 0.02° .

Differential scanning calorimetry

The thermal properties of TCP0 and TCP40 were assessed by differential scanning calorimetry (DSC, TA Instruments Q2000 DSC). The samples were heated from $10\text{ }^{\circ}\text{C}$ to $200\text{ }^{\circ}\text{C}$ at a rate of $10^{\circ}\text{C}\cdot\text{min}^{-1}$ and held isothermal for 10 min to remove thermal history. Then, the samples were cooled to $-30\text{ }^{\circ}\text{C}$ at a rate of $10^{\circ}\text{C}\cdot\text{min}^{-1}$, held isothermal for 10 min, before heating them again to $200\text{ }^{\circ}\text{C}$ at $10^{\circ}\text{C}\cdot\text{min}^{-1}$. The melting temperature was determined from the second heating cycle.

Scaffold design and fabrication

3D models of scaffolds with two different pore shape architectures, S and D shapes, were designed. The G-code to fabricate a cylindrical scaffold with S-shaped pores was generated in the PrimCam software of the printer. The S-shaped pores were created by

rotating the deposition angle after every layer by 15 degrees. The strand distance and the layer thickness were set at 850 μm and 200 μm , respectively.

The D 3D model was designed in the Rhinoceros software with the Grasshopper plugin, based on the equation (1) of the diamond TPMS [34].

$$\sin(x)\sin(y)\sin(z) + \sin(x)\cos(y)\cos(z) + \cos(x)\sin(y)\cos(z) + \cos(x)\cos(y)\sin(z) = 0 \quad (1)$$

The 3D model was sliced with a custom-made slicing code, setting a layer thickness of 200 μm , and the G-code was generated with a custom-made G-code generator. Both scaffold shapes were designed to have a theoretical porosity of 70%.

All scaffolds were fabricated with an extrusion-based printing system (Bioscaffolder, SYSENG, Germany). TCP0 or TCP40 were added in the reservoir, capped with a Teflon plunger, and melted at $T = 100\text{ }^{\circ}\text{C}$ or $T = 140\text{ }^{\circ}\text{C}$, respectively. The materials were extruded through a 25G nozzle, at a pressure of 5 bar, a screw rotation of 11 rpm, and a translation speed of $100\text{ mm}\cdot\text{min}^{-1}$. Cylindrical scaffolds for mechanical testing were printed with a diameter of 6 mm and a height of 4 mm. Samples for cell studies had a diameter of 6 mm and a height of 2 mm.

Morphological characterization

SEM

The morphology of the scaffolds was observed using a Jeol JSM-IT200 InTouchScope (Tokyo, Japan) SEM, with a voltage of 10 kV and a working distance of 10 mm. Before imaging, the scaffolds were coated with gold, using a Cressington sputter coater 108 Auto. Fiber diameter, strand distance, and layer thickness were measured from the images using the Fiji Software.

Micro-computed tomography

Micro-computed tomography (micro-CT) was performed using a SkyScan1272 (Bruker, Kontich, Belgium) scanner. TCP0 samples were scanned at a pixel size of 3.5 μm , 50 kV and 150 μA , rotation step of 0.2° , and 4 averaging frames, while for TCP40 samples a 0.25Al filter was used ($n=3$). Images were reconstructed using NRecon software (1.7.1.6, Bruker) and samples were visualized using CTvox (3.3.0 r1403, Bruker) and DataViewer (1.5.4.6, Bruker). The quantitative data was obtained using CTan software 1.18.4.0+, Bruker).

Mechanical characterization

The mechanical performance of the scaffolds was determined upon compression using a TA ElectroForce system (TA Instruments), equipped with a 450 N load cell, and controlled with Wint7 software. The tests were conducted at a strain rate of $0.04\text{ mm}\cdot\text{s}^{-1}$ up to a

maximum of 50% compression. TCP0 and TCP40 scaffolds with both S and D shape were tested (n=5). Before testing, the height and the diameter of the samples were measured with a caliper, and the scaffolds were weighted to calculate the net porous area, with the formula:

$$A = \frac{m}{h \times \rho}$$

Where:

m is the weight of the sample;

h is the height of the sample;

ρ is the density of the raw materials (TCP0 = 1.145 g·cm⁻³, TCP40 = 3.14 g·cm⁻³).

The stress values were obtained dividing the measured load against the area of the fibers for each scaffold [35]. The compressive modulus was calculated between approximately 5-15% strain in the linear region of the stress-strain curves. The yield stress and strain were calculated with the 0.2% offset method from the stress-strain curves. The toughness was calculated as the area under the stress-strain curves in the plastic region, defined from the yield points.

Bioactivity evaluation

Simulated body fluid

The bioactivity of the composite scaffolds was tested in simulated body fluid (SBF) 1X solution, for 7 and 28 days. The solution was prepared following Kokubo's method [36], as reported in the Supplementary Information (**Table S1**). All scaffolds were pre-wetted in MilliQ water and placed in a 24-well plate with 2 mL of SBF solution. The samples were incubated at T = 37 °C and the solution was refreshed every 2-3 days. After 7 and 28 days, the samples were washed 5 times in MilliQ water and dried overnight. Samples were gold coated, imaged on the SEM, and EDX (elemental) analysis was performed.

Ion release in culture medium

TCP40 scaffolds were incubated in cell culture medium for 28 days and the Ca and P release was quantified by inductively coupled plasma mass spectrometry (ICP-MS, iCAP Q, Thermo Scientific) in standard mode (n=3). At different time points, 100 µL of sample were dissolved in 4.9 mL of 1% nitric acid, containing Scandium at a concentration of 200 ppb as internal standard. The standard curve was obtained in the range 0.3-4.8 ppm.

hMSCs culture and differentiation

hMSCs were purchased from PromoCell (donor Caucasian woman, 30 years old). Cells were expanded in T225 flasks at a density of 1000 cells·cm⁻² in α - minimum essential medium (α -MEM) (Gibco) supplemented with 10% v/v fetal bovine serum (FBS). Cells were passaged at 70-80% confluency and used at passage 5 for experiments.

Scaffold seeding

For cell experiments, TCP0 and TCP40 scaffolds with S or D shapes were printed. Prior to seeding, the scaffolds were sterilized in 70% ethanol for 30 min, washed 3 times in phosphate buffer (PBS), and incubated overnight at 37 °C in basic culture medium (BM). The BM consisted of α -MEM supplemented with 10% v/v FBS, 0.2 mM l-ascorbic acid-2-phosphate, 100 U·mL⁻¹ penicillin-streptomycin.

200'000 cells were seeded at passage 5 in BM with the addition of 10% w/v of dextran (Pharmacosmos, 5510 0500 9006, Denmark) per scaffold. After 24 h (day 1), the media was changed to either BM, or osteogenic medium (OM = BM + 10 nM dexamethasone), or mineralization medium (MM = BM + 10 nM dexamethasone + 10 mM beta-glycerophosphate). Scaffolds were kept in culture for 28 days and medium refreshed every 2-3 days.

Immunostaining

Immunostaining was performed after day 1, 7, and 28. At each time point samples were fixed in 4% paraformaldehyde (PFA), washed and stored in PBS (n=3). Then, the cells were permeabilized for 20 min in 0.1% Triton X-100 (Merck) and rinsed again 3 times in PBS. The blocking was done for 1.5 h in 3% Bovine Serum Albumin and 0.01% Triton X-100. Samples were then incubated overnight at 4 °C in dark with phalloidin and primary antibodies, depending on the fixation time point, as summarized in **Table 1**.

After incubation, primary antibodies and phalloidin were three times washed with 0.3% BSA, 0.01% Triton X-100 in PBS. Phalloidin was used to stain cells actin filaments. Secondary antibodies (goat anti-rabbit and goat anti-mouse, 1:300, Abcam) were added to the samples and incubated in dark for 45 min. Finally, samples were stained with DAPI (1:300) in dark for 20 min and rinsed with PBS. Stained samples were imaged with a Leica TCS SP8 CARS confocal microscope.

Table 1. Constructs were stained after 1, 7, and 28 days with primary antibodies and/or phalloidin.

Time point	Antibody/Phalloidin
Day 1	Alexa Fluor Phalloidin 647 (1:100, Thermo Fisher)
Day 7	Alexa Fluor Phalloidin 647 (1:100, Thermo Fisher) Rabbit anti-Runx2 (1:250, ab192256, Abcam) Mouse anti-Sox9 (1:200, ab76997, Abcam)
Day 28	Alexa Fluor Phalloidin 647 (1:100, Thermo Fisher) Rabbit anti-osteocalcin (1:250, ab93876, Abcam) Mouse anti-collagen I (1:300, ab6308, Abcam)

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was quantified for all the samples on day 7 and day 28 (n=5). The scaffolds were washed twice with PBS, transferred in Eppendorf tubes and stored at -80 °C. Then, three cycles of thawing at room temperature (RT) and freezing in liquid nitrogen were performed, and samples were incubated in a lysis buffer (0.1 M KH_2PO_4 , 0.1 M K_2HPO_4 , 0.1 % Triton X-100, pH 7.8) for 1 h at RT. At this point, 50 μl of lysates were transferred in a different Eppendorf and used to perform ALP activity assay. Briefly, 10 μl of lysate was transferred in duplicate in a white 96-well plate. Then, 40 μl of a CDP-star chemiluminescent substrate reagent (ready-to-use, Roche) were added to the samples and incubated for 15 min at RT in dark. The luminescence was measured at 466 nm using a CLARIOstar spectrophotometer (BMG Labtech). The results of the ALP activity quantification were normalized against the available surface area or cell density, calculated as the total DNA divided by the pore volume (see section 2.6.4). The remaining cell lysate was used for DNA quantification and hydroxyproline (HYP) assay.

DNA quantification

To evaluate the seeding efficiency and the proliferation of the cells, the DNA was quantified at day 1, 7 and 28 (n=5). To further digest extracellular matrix, 200 μl of cell lysates from ALP activity were incubated overnight at 52 °C in 200 μl of 1 $\text{mg}\cdot\text{mL}^{-1}$ proteinase K, dissolved in solution comprising 50 mM Tris, 1 mM ethylenediaminetetraacetic acid, and 1 mM iodoacetamide (TRIS/EDTA). From the digested samples, 100 μL were taken for HYP assay. At this point, three freeze-thawing cycles were performed, and the CyQUANT™ Cell Proliferation Assay kit (Thermo Fisher) was used for a DNA assay. Briefly, scaffolds were incubated at RT in 300 μl of lysis buffer containing DNase-free RNase A (1:500) (Thermo Fisher). Then, 100 μl of each sample was transferred in a 96-well plate in duplicate and incubated at RT with 100 μl of a 2x GR-dye solution for 12 min. A standard curve was made with a DNA standard, and the fluorescence was measured at $\lambda_{\text{ex}} = 480 \text{ nm}$ and $\lambda_{\text{em}} = 520 \text{ nm}$ with a CLARIOstar spectrophotometer (BMG Labtech).

At Day 1, the seeding efficiency was estimated from the measured DNA values, assuming a DNA content per cell of 6.6 pg DNA $\cdot\text{cell}^{-1}$. For each scaffold shape, the DNA values were normalized against the available surface area or against the pore volume obtained from micro-CT measurement, to calculate the cell density.

Collagen production

Proteinase K digested samples (see section 2.6.4) were diluted 1:4 at day 7 and 1:5 at day 28 in TRIS/EDTA solution and hydrolyzed 1:1 in 37% HCl for 18 h at 110 °C (n=5). Then, the solvent was evaporated in a vented oven, and the hydrolysis product was suspended in 200 μl of MilliQ water. The HYP was oxidized in a chloramine T containing

buffer for 20 min at RT. Then, the product was made fluorescent by reaction with 4-dimethylaminobenzaldehyde. A standard curve was prepared by serial dilutions of trans-4-hydroxy-L-proline solution. Absorbance was measured at 570 nm. The total collagen content was calculated assuming a HYP to collagen ratio of 1:7.69, and the values were normalized against the available surface area or the cell density for each scaffold.

ECM morphology and mineralization assay

At day 7 and 28, the scaffolds in BM and OM were fixed with 4% PFA for 30 min and washed three times with PBS. Then, the samples were dehydrated in a series of ethanol solutions (30, 50, 70, 80, 90, 96 and 100% in water) for 15 min at RT. Finally, the samples were immersed in a series of absolute ethanol and hexamethyldisilazane (HMDS) solutions (72:25, 50:50, 25:75, 0:100) for 15 min. At the end, the samples were dried overnight at RT to remove residual HMDS. Samples were sputter coated with gold and imaged via SEM.

Calcium deposition was observed with an Alizarin Red staining (n=3). Samples in BM and MM at day 28 were fixed in 4% PFA for 30 min and washed with distilled water. Alizarin red solution (ARS) 40 mM was prepared in distilled water and the pH was adjusted at 4.1-4.3. The scaffolds were incubated in the solution for 20 min at RT and then washed thoroughly until the ARS was completely removed. The stained samples were imaged with Stereomicroscope (NIKON SMZ25) for a qualitative evaluation of calcium deposition.

Moreover, the stained samples were placed in Eppendorf and incubated with 400 μ l of 10% chloride monohydrate (CPC) in 10 mM sodium phosphate (pH=7) overnight, to completely extract the dye. A standard curve was made with a serial dilution of the ARS in 10% CPC. Finally, 100 μ l of each sample were transferred in duplicates in a 96-well plate and measured at 562 nm using a CLARIOstar spectrophotometer. Acellular scaffolds served as blanks to subtract the background values originating from the TCP inherently present in the TCP40 scaffolds. The results were normalized against the available surface area, or the pore volume of each scaffold measured with micro-CT.

Gene expression

Gene expression was evaluated at day 7 and day 28 on scaffolds in BM and OM (n=3). At each time point, were minced, collected in 1 ml of Trizol (Invitrogen), and stored at -80 °C. After three steps of freeze-thawing, the scaffolds were centrifuged at 12000 g for 5 min at 4 °C to remove scaffold pieces and precipitate ECM. The supernatant was then transferred into new Eppendorf and 200 μ l, mixed with chloroform, and centrifuged at 12000 g for 15 min at 4 °C. The aqueous phase was collected, and 500 μ l of isopropanol was added, and centrifuged for 10 min at 12000 g at 4 °C. The supernatant was discarded, and the precipitated RNA was re-suspended in RNase-free water. The RNA concentration, and the 260/280 and 260/230 values were measured using Bio-drop uLITE instrument

(Scientific Laboratory Supplies). cDNA was synthesized from 200 ng of RNA, using iScript cDNA synthesis kit (Bio-Rad, California, USA), following the manufacturer protocol. Finally, the qPCR reactions were conducted in 20 μ l of volume containing 4 ng of cDNA in 2 μ l of RNase-free water, 10 μ l of iQ SYBER green Supermix (Bio-Rad) and 8 μ l of reverse and forward primer mix (**Table S2**). A CFX96™ IVD RT-PCR system (Bio-Rad) was used with a thermal cycle of 95 °C for 3 min, 95 °C for 15 s and 55 °C for 20 s for 40 cycles. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative gene expression [37]. B2M was chosen as housekeeping gene and values were normalized with reference to the relative expression of cells cultured on TCP0_S scaffolds at day 7 in BM or OM.

Statistical analysis

All the quantitative data are shown as means $\pm$ standard deviation. The statistical analysis was performed with the software GraphPad Prism. Three to five replicates were used for each experiment. The normality of the data was assessed with a Shapiro-Wilk test, and when confirmed, statistical significance was determined with parametric student t-test or ANOVA. Otherwise, non-parametric tests were done. (****) $p < 0.0001$, (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$.

Results and discussion

Composite preparation and characterization

Ball milling of the particles allowed to obtain a β -TCP particle size of 1223 ± 98.2 nm (**Figure S1A, B**). Morphological features of the particles, such as size, microstructure, porosity, and crystallinity, are known to tune the bioactive potential of β -TCP [38, 39]. Therefore, we targeted a sub-micron particle size range, since it was demonstrated to induce higher osteoclast activity and hMSCs differentiation [39, 40].

A blending strategy was used to produce TCP40. The TGA results displayed an amount of TCP of $41.97 \pm 0.45\%$, implying high efficiency, limited loss of material, and reproducibility of the method (**Figure 1B**). The incorporation of the TCP particles did not affect the T_m of PCL, which remained around 55 °C, but a small reduction in the polymer crystallinity was observed as indicated by the enthalpy of the melting peak (**Figure S1C**).

The chemical composition of TCP40 was investigated with both FTIR and XRD, in comparison with TCP0 and the TCP particles alone. FTIR analysis (**Figure 1C**) showed that both the TCP0 and the TCP40 scaffolds presented the typical bands associated with the polymeric chains [41]. The CH₂ symmetric and asymmetric vibration stretches are located at 2868 cm⁻¹ and 2945 cm⁻¹, respectively. The C=O stretching is found at 1728 cm⁻¹, and the C-O stretch mode at 1290 cm⁻¹. Finally, the C-O-C stretching of the ether is found at 1165 cm⁻¹. As expected, in both the TCP and the TCP40 the characteristic band of the

asymmetric stretching and bending of the PO_4^{3-} were found at 1013 cm^{-1} and 540 cm^{-1} , respectively [41, 42].

Furthermore, XRD diffraction patterns showed the typical sharp peaks of the β -TCP in the powder only, at $2\theta = 27^\circ$, $2\theta = 30.3^\circ$, and $2\theta = 33.6^\circ$ (**Figure 1D**), corresponding to the (214), (015), and (220) planes, respectively [43]. In the TCP40, the TCP pattern was maintained, but amorphous peaks appeared, due to the presence of the polymer (Δ), at $2\theta = 21.4^\circ$ and 23.7° , corresponding to (110) and (200) crystallographic planes, respectively [44].

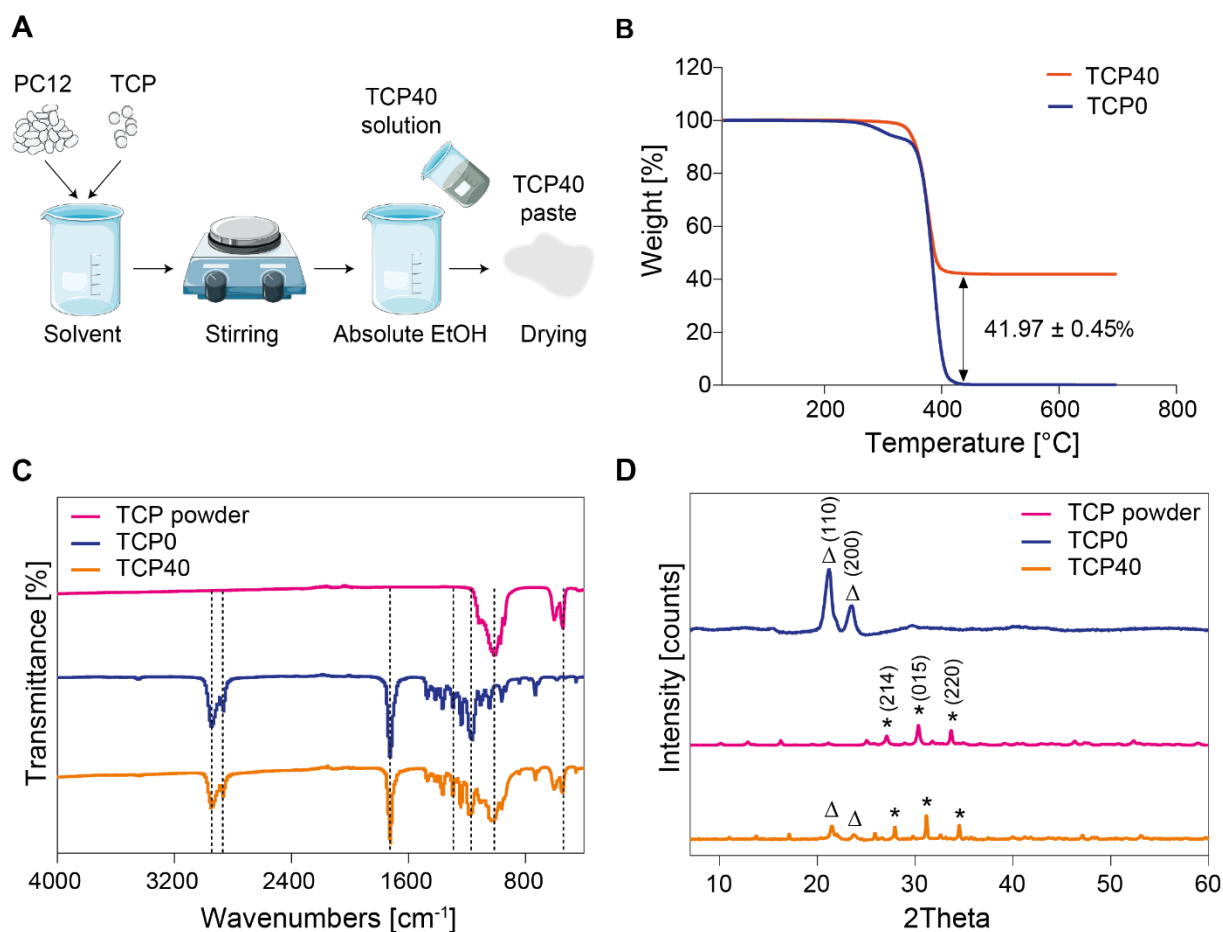


Figure 1. Schematics of the composite production process (A). Representative TGA curves of the TCP40 and TCP0 samples (B). FTIR (C) and XRD (D) of the TCP40 composite in comparison with TCP0 and TCP powder. The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 license.

Scaffold fabrication and morphological and mechanical characterization

TCP0 and TCP40 scaffolds were fabricated with a S- and D- pore shape architecture, according to the .STL designed files depicted in **Figure 2A**. SEM images revealed that S- and D-like pore shapes had a fiber diameter and layer thickness of approximately $250\text{ }\mu\text{m}$ and $200\text{ }\mu\text{m}$, respectively (**Figure 2B**, **Figure S2A-B**). Moreover, the map of elements obtained from SEM images revealed a homogeneous distribution of the Ca and P on both the surface and the cross section of the TCP40 fibers (**Figure S2C**).

In addition to SEM, morphological evaluations were performed via micro-CT analysis. Images of the cross-sections indicated a high printing fidelity of both scaffold architectures (**Figure 2C, Figure S3**). The S scaffolds displayed a higher surface area than D scaffolds (522.9 ± 17.6 vs 446.2 ± 4.3 mm²). Conversely, the pore volume of D scaffolds was higher than the S ones (51.8 ± 2.5 vs 43.5 ± 0.7 mm³). The differences in pore volume can also be qualitatively appreciated from the videos recorded with micro-CT, which revealed that the S pores are more interconnected (**Supplementary videos S1 and S2**). Moreover, the experimental porosity calculated with the micro-CT was on average 66%, close to the theoretical one of 70% set in the 3D models.

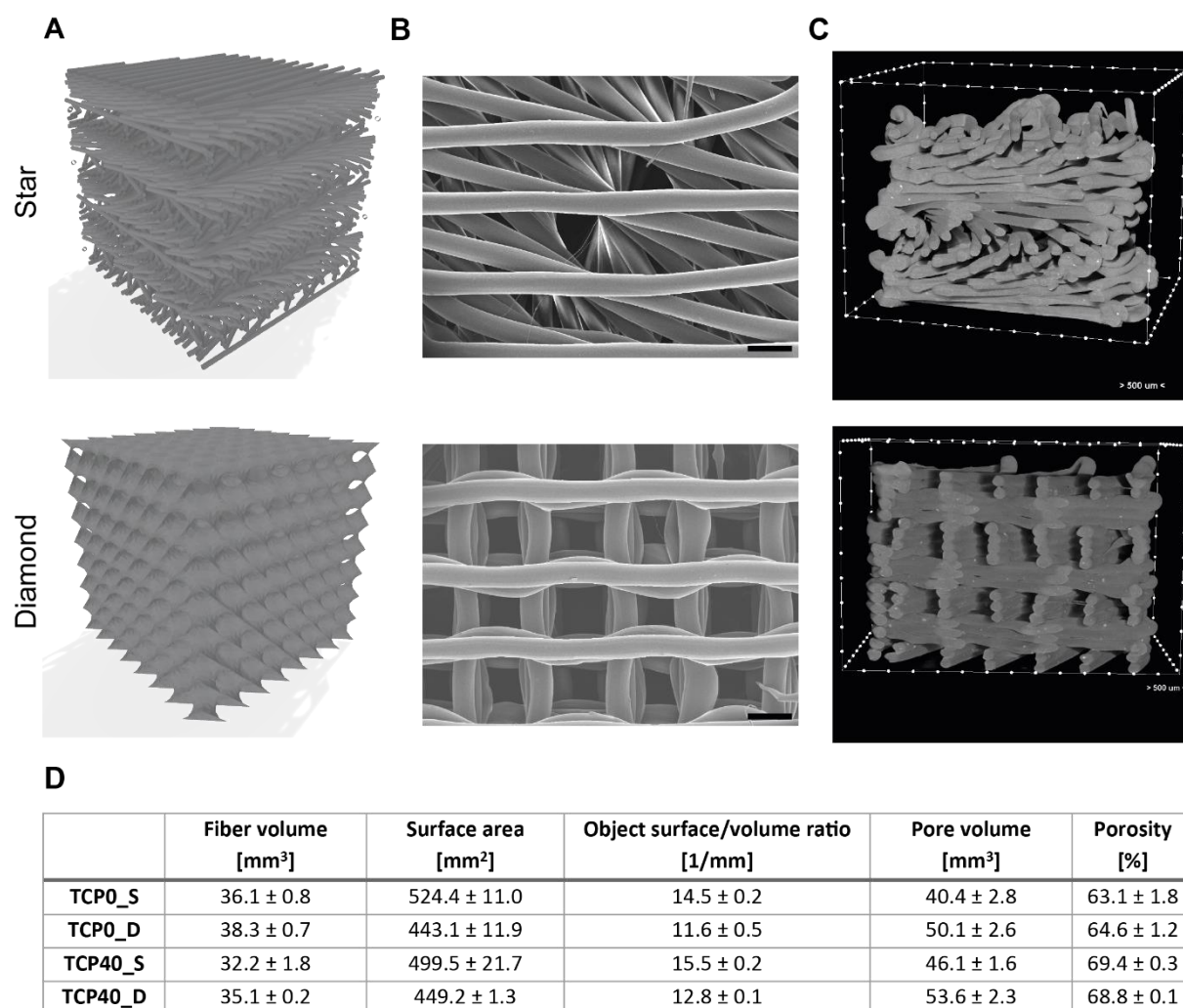


Figure 2. 3D models of the S (top) and D (bottom) scaffolds (A). SEM images of the top view of the 3D printed S (top) and D (bottom) scaffolds. Scale bar = 500 μ m (B). Micro-CT images of the S (top) and D (bottom) 3D printed TCP40 scaffolds (C). Morphological parameters of the TCP0 and TCP40 scaffolds with S and D shape, measured with micro-CT (D).

The mechanical performance of the scaffolds, comprising different shapes and material composition, was tested under compression. The stress-strain curves presented an initial elastic response up to approximately 15% strain (**Figure 3A**). Notably, in the linear region

of the stress-strain curves, the D-shaped scaffolds consistently displayed higher compressive moduli compared to their S-shaped counterparts, a trend observed for both TCP0 (18 ± 2 and 103 ± 8 MPa for S and D, respectively) and TCP40 scaffolds (43 ± 7 and 277 ± 16 MPa for S and D, respectively) (**Figure 3B**). We attributed the differences between the two shapes to the different amount of contact points between consecutive fibers, which can also be observed from the micro-CT scans of the cross-sections (**Supplementary video S1 and S2**). Particularly, the S scaffolds present lower contacts between consecutive layers because of the rotation of 15° , resulting in a weakening of the structure, as also previously observed in other studies [13, 27]. The D model, instead, is characterized by the repetition of 3 wave-like layers of fibers deposited along the same direction, thus reinforcing the structure. Moreover, the TCP40 scaffolds displayed a higher compressive modulus than the corresponding TCP0, which is likely a result of reinforcement effects of the TCP particles [41, 45, 46].

Subsequently, other mechanical parameters were calculated from the stress-strain curves (**Figure S4A – D**). The yield strength was significantly higher for the D constructs, regardless the material type (8.9 ± 0.3 and 15.4 ± 2.1 MPa, for TCP0_D and TCP40_D, respectively). All the samples presented a yield strain of approximately 15%. Finally, the toughness was significantly higher for the D samples compared to the S ones (432.7 ± 27.7 and 760.3 ± 74.5 N·mm², for TCP0_D and TCP40_D, respectively).

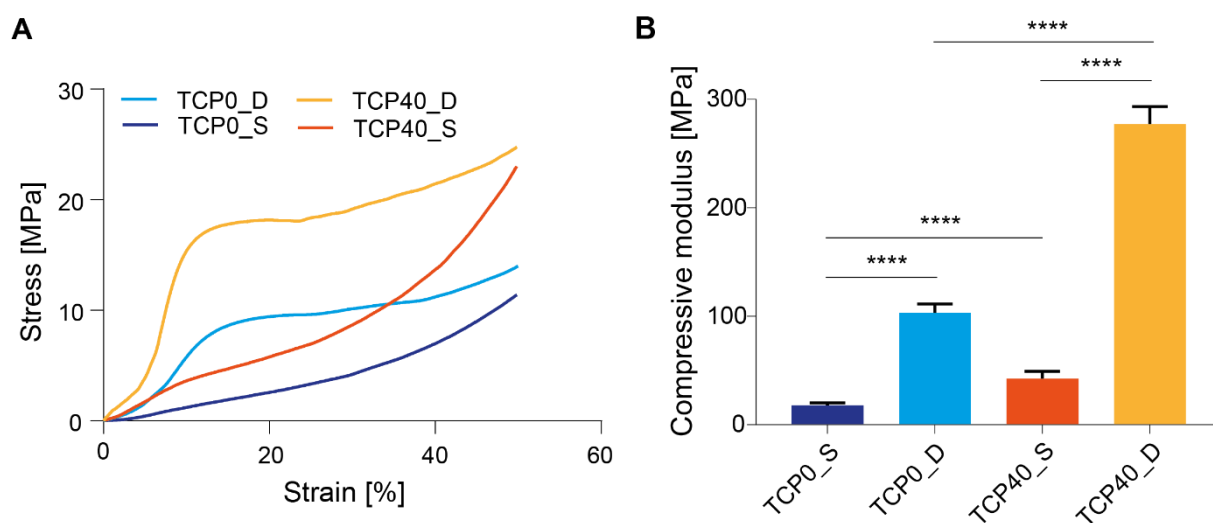


Figure 3. Stress-strain curves of the TCP0 and TCP40 with S or D shape tested under compression, n=5 (A). Compressive moduli for TCP0 and TCP40 with S or D shapes, n=5 (B).

Bioactivity

Since EDX analysis on the TCP40 scaffolds revealed the presence of Ca and P elements (**Figure 4A**), we evaluated the bioactivity of the constructs at day 7 and 28. First, the samples were immersed in SBF, a solution that mimics the ion concentrations of human blood plasma [36, 47]. After 7 days, on both TCP0 and TCP40 scaffolds no differences were

observed compared to the scaffolds before incubation (**Figure S5A**). However, by day 28, significant mineral deposition occurred on the TCP40 surfaces of both S and D scaffolds, with no qualitative differences between the two (**Figure 4B**). Notably, the EDX analysis at this time point did not display the C elements characteristic of the polymeric component, suggesting complete coverage of the scaffold surfaces by mineral deposits. At the same time point, no mineral deposition occurred on the TCP0 scaffolds (**Figure S5B**).

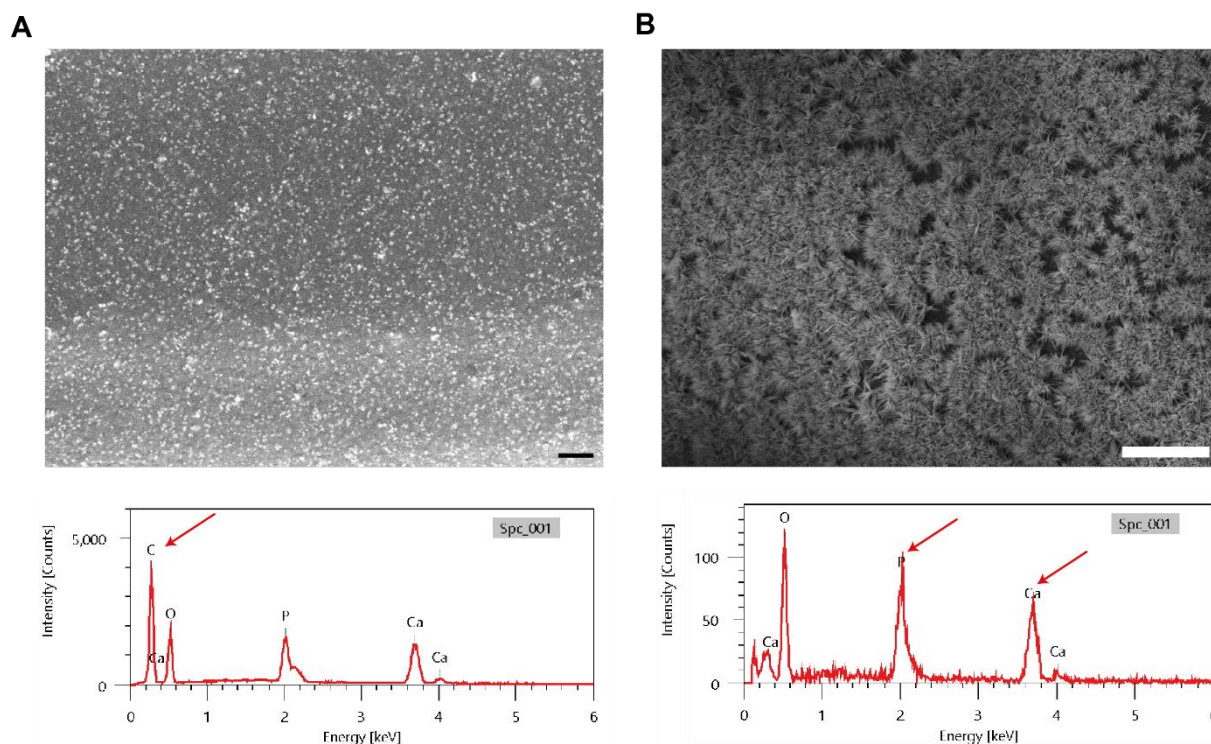


Figure 4. Representative SEM image of a TCP40_D scaffold and EDX analysis before (A) and after 28 days of immersion in SBF solution (B). Scale bar = 10 μm.

We hypothesized that the presence of TCP in the TCP40 scaffolds played a role in the bioactivity of the constructs. However, mineral deposition in SBF did not occur during the initial days of immersion, requiring a longer period. We hypothesized that this was due to the formation of a thin polymer layer over the fibers. Over time, the polymer could have gradually degraded, exposing the particles to the surface. To prove this, we cut the scaffolds in half, thus exposing the particles of the cross section directly to the solution. After 7 days we observed the samples at SEM and found mineral deposition already at this early time point, confirming the hypothesis (**Figure S5C**). Moreover, similar observations have been documented in literature [40, 48, 49]. In this context, surface alkaline etching has been proposed as a strategy to accelerate surface erosion and, consequently, particle exposure [50, 51].

Subsequently, we investigated the release dynamics of Ca and P in cell culture conditions. TCP0 and TCP40 scaffolds were incubated in culture medium, which was

analyzed at different time points. ICP-MS analysis revealed that at each time point the ionic concentration of both Ca and P was similar between the samples (**Figure S5D**). However, since the measured concentrations were comparable to the blank (culture medium only), the data suggested that no particles were released from the scaffolds.

Similar to what happened in SBF, we believe that after 28 days the degradation of the first polymeric layer occurred. However, the TCP particles were not dissolved in the medium, because of their low solubility in non-acidic solutions, thus remaining on the surfaces. Indeed, SEM images showed fully exposed particles to the external environment but still adhered on the surface of the scaffolds (**Figure S5E**).

Cell adhesion and proliferation

After scaffold characterization and assessing the mineralization potential, we wanted to investigate cell adhesion and proliferation of hMSCs on TCP0 and TCP40, D or S-shaped, over 28 days of culture. First, we evaluated whether the scaffold architecture significantly affected the cell seeding efficiency by assessing the cell number and distribution. Actin staining revealed that the cells were homogeneously dispersed throughout the scaffolds, similarly for all the different samples (**Figure S6A**). Moreover, we quantified the DNA content and the number of cells after 24h. To compare the effect of the shape, the values were normalized against the available surface area of each shape, being the cells mostly present on the surface of the fibers at early time points (**Figure S6B**). The seeding efficiency of the scaffolds was approximately 44%. This relatively high value was attributed to the seeding procedure with dextran-supplemented media [52]. Moreover, no significant differences in the seeding efficiency were observed comparing the samples. This was also attributed to the dextran seeding procedure, which might mask the initial effect of the shape. In fact, it was observed before that in scaffolds seeded without dextran the S shape resulted in higher and more homogenous seeding efficiency compared to other standard shapes, like woodpile and triangular pore architectures [13].

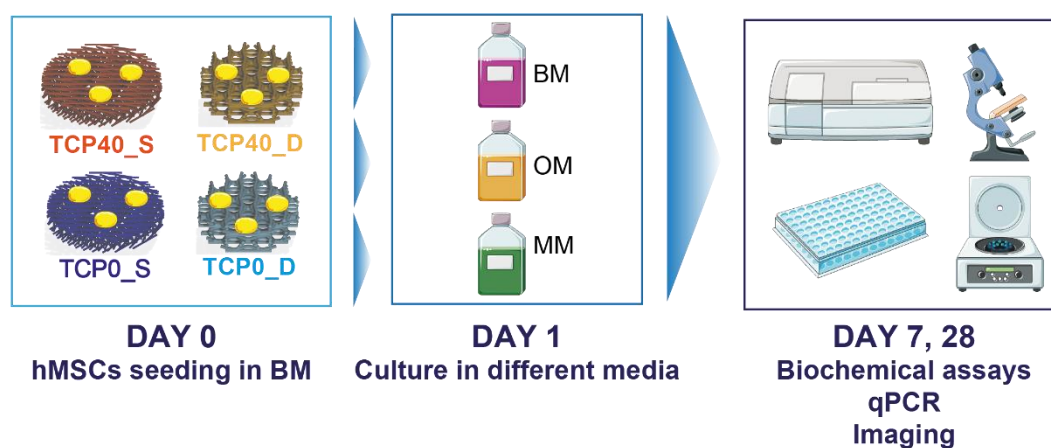
At day 7 and day 28, the cell density was calculated to assess proliferation. Here, the DNA values were normalized against the pore volume, since the pores were already filled with cells (**Figure S6C**). Both in BM and OM, the cells proliferated over time, with no significant differences between the conditions (**Figure S6D-E**). From here on, the biological data is always normalized on the pore volume. However, for completeness, we reported also the data normalized against surface area in Supplementary **Figure S7**.

hMSCs differentiation

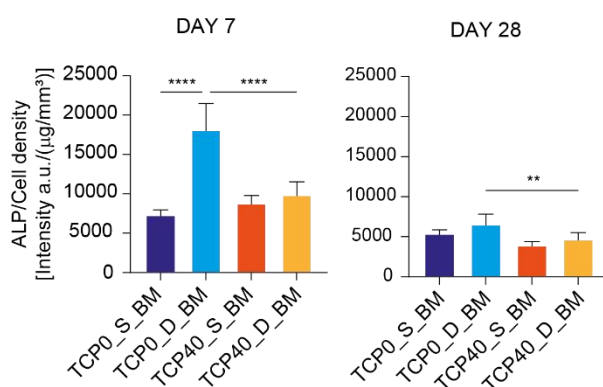
After investigating the capacity of the developed scaffolds to support cell adhesion and proliferation, we evaluated the effect of scaffold composition and shape on osteogenic differentiation of hMSCs over 28 days, which is considered as a sufficient late time point

to observe mature osteogenic differentiation in vitro [53-55]. The cells were seeded on TCP40 and TCP0 scaffolds with D or S shape, and after 24h the constructs were incubated in basic, osteogenic, or mineralization medium. First, at day 7, we assessed the expression of master transcription regulators for osteogenesis and chondrogenesis, Runt-related transcription factor 2 (Runx2) and SRY-Box Transcription Factor 9 (Sox9), respectively, as hMSCs can differentiate in both phenotypes [56, 57]. Confocal images displayed higher presence of RUNX2 localized in the cell nuclei, compared to SOX9 (**Figure S8**). Early upregulation of Runx2 and inactivity of Sox9 indicated the commitment of hMSCs towards an osteogenic phenotype [58-60]. Therefore, after 7 and 28 days, osteogenic differentiation markers were investigated at a molecular and protein level (**Figure 5A**).

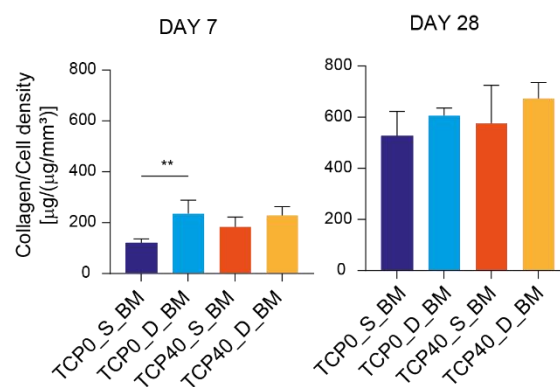
A



B



C



D

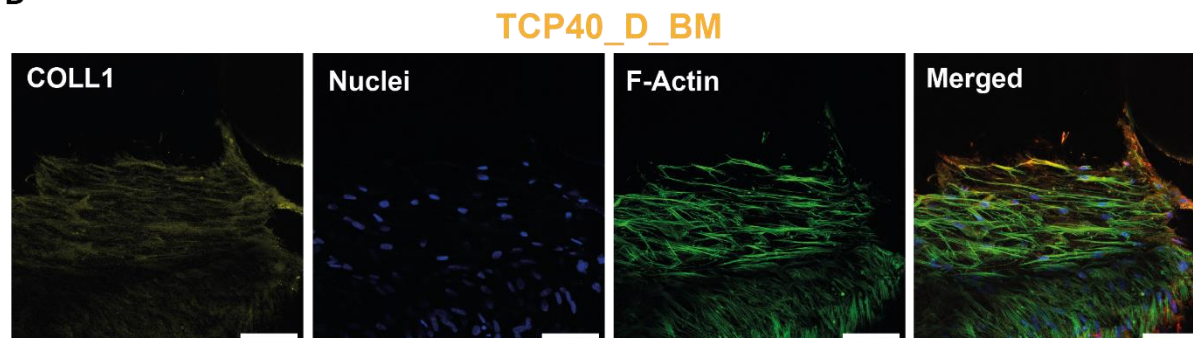


Figure 5. Schematic representation of the experiment. At day 0 the cells are seeded on all the scaffolds in BM. At day 1, the scaffolds are divided in 3 groups: BM, OM (BM + Dexamethasone), and MM (BM + Dexamethasone + β -glycerophosphate (A). Normalized ALP activity at day 7 and day 28 in BM, n=5 (B). Normalized collagen production at day 7 and day 28 in BM, n=5 (C). Representative confocal images of TCP40_D scaffolds at day 28 in BM, stained for Collagen I (yellow), DAPI (blue), and F-actin (green). Scale bar = 200 μ m (D). The Figure was partly generated using Servier Medical Art, provided by Servier, licensed under a Creative Commons Attribution 3.0 unported license.

ALP activity

As an early marker of osteogenic differentiation, the normalized ALP activity was measured after 7 and 28 days of culture, in both BM and OM. In BM, ALP activity was shown to be higher at day 7 than at day 28 (**Figure 5B**). Interestingly, we could observe higher values of the D scaffolds ($18.2 \pm 3.3 \cdot 10^3$ and $9.9 \pm 1.6 \cdot 10^3$ intensity a.u./ μ g/mm³, for TCP0 and TCP40, respectively), when comparing them to the respective S ones, suggesting a possible effect of the shape on ALP activity. In OM, the trend remained the same, but with overall higher values (**Figure S9A**).

No significant effect of the TCP40 samples on cell differentiation was found compared to TCP0 scaffolds. Similar observations were found in literature, where calcium phosphate-based composites did not show significant improvement of ALP activity [61, 62]. This was ascribed to a slow ion release dynamic, leading to insufficient Ca²⁺ concentration in the medium so to affect Ca²⁺ signaling pathways involved in osteogenesis.

ECM morphology and collagen quantification

The ECM formation on the scaffolds was assessed using SEM at day 7 and 28 (**Figure S9B**). Cross-sectional images revealed that cells were homogeneously distributed across the scaffolds, with no qualitative differences between all conditions. While at day 7 the pores appeared still open, at day 28 a much higher matrix deposition could be observed (**Figure S9B**).

The production of collagen was measured since it is the main component of the bone ECM. After 28 days in BM, a roughly 5 times higher amount of collagen was produced compared to day 7 (**Figure 5C**). Like the trends observed in ALP activity, at both day 7 and 28 the D scaffolds showed higher values compared to the respective S ones. At day 7, TCP0_D and TCP0_S were 234.9 ± 53.1 and 120.1 ± 15.5 μ g/(μ g/mm³), respectively. When we compared the material effect, we observed higher collagen production for the TCP40 scaffolds. At day 28, TCP40_D and TCP0_D displayed a value of 673.1 ± 63.47 and 605.4 ± 30.5 μ g/(μ g/mm³), respectively. Similar trends were observed in OM (**Figure S9C**). Additionally, immunostaining at day 28 revealed abundant presence of Collagen I in the secreted matrix, without qualitative differences between the samples, proving that bone ECM was deposited (**Figure 5D, Figure S9D**).

Mineralization

After having confirmed the deposition of bone collagenous matrix, we assessed mineralization after 28 days of culture. Since alizarin red staining (ARS) binds Ca deposits [63], acellular composite scaffolds were also incubated in media to remove the background values of the scaffolds from the samples (**Figure S10A**). We found that in the TCP40 scaffolds the matrix mineralized, whereas TCP0 scaffold appeared completely unstained (**Figure 6A, Figure S10B**). In BM, the quantification of the staining showed that the TCP40_S samples were significantly higher compared to TCP40_D (15.4 ± 0.4 and 13.6 ± 0.7 mM/($\mu\text{g}/\text{mm}^3$), respectively) (**Figure 6B**). We attributed this to the greater surface area of the S scaffolds compared to the D ones, which probably led to higher area of the exposed CaP particles on the surface of TCP40 scaffolds.

However, the material composition had a more predominant effect on mineralization than the pore shape. After 28 days, the thin PCL layer initially covering the particles could have slowly degraded, resulting in particle exposition, as previously discussed (Section 3.3) and reported in literature [61]. In MM the scaffolds displayed the same behavior (**Figure S10C**).

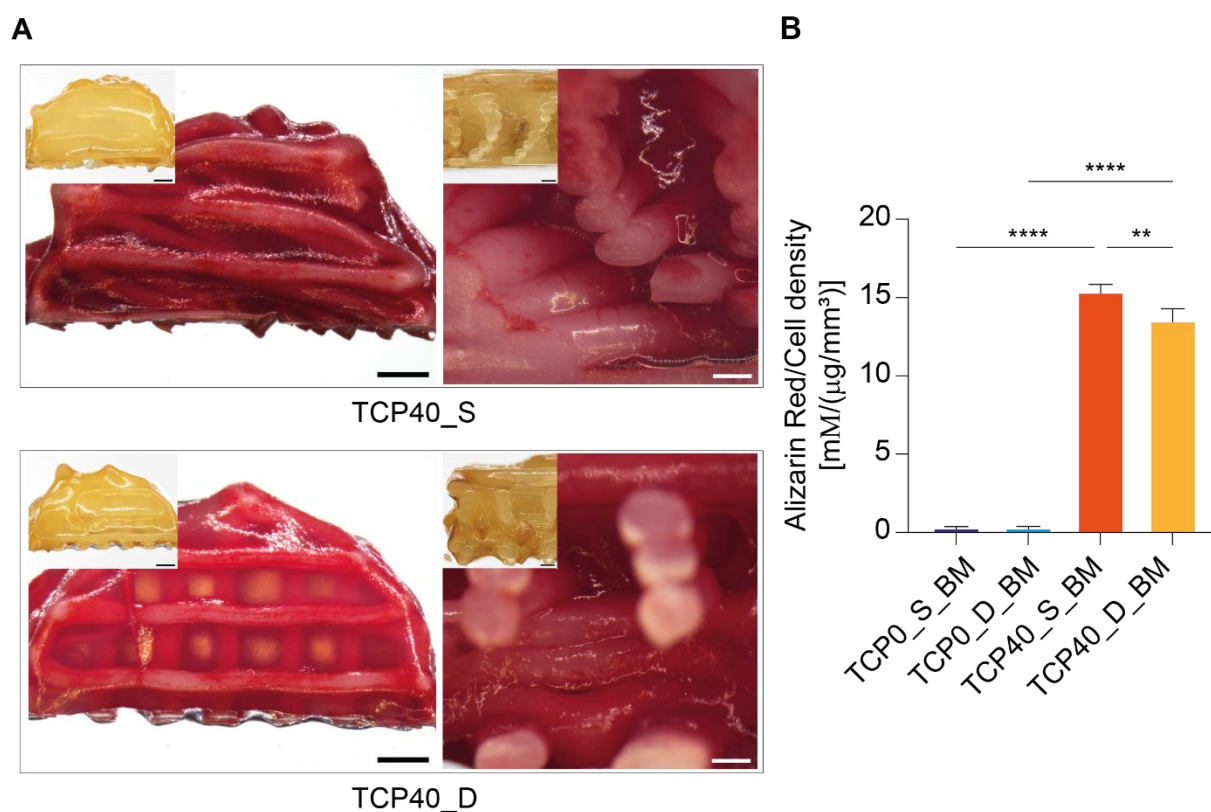


Figure 6. Stereomicroscope images of ARS at day 28 in BM, for TCP40_S and TCP40_D samples, from the top and of the cross section. In the up-left corner, the corresponding TCP0 scaffolds. Scale bar = 200 μm (A). ARS quantification at day 28 in BM, $n=3$ (B).

Gene expression and immunostaining

As matrix mineralization implied successful progression in osteogenic differentiation, we assessed the expression of osteogenesis-associated genes at day 7 and 28 in BM

(**Figure 7**) and OM (**Figure S11A-B**). The gene activity was normalized to the values of TCP0_S on day 7.

Since they play a pivotal role in the early phases of osteoblastic differentiation, we assessed RUNX2 and ALP gene expression (**Figure 7A**). At day 7, both genes were already expressed with an average fold change of around 1. After 28 days, RUNX2 expression remained constant among all samples, except for a small decrease observed in the TCP40_D construct, whereas ALP expression was 10-fold downregulated. Bone morphogenic protein 2 (BMP2) and Collagen 1A1 (COLL1A1) expression were investigated, as they are typically activated during the middle stage of the osteogenic differentiation and are involved in promoting matrix production and remodeling[64-66]. After 28 days, both genes displayed a 1.5-2-fold upregulation compared to day 7 (**Figure 7B**). Moreover, while minor differences were observed between the shapes, TCP40 scaffolds presented higher BMP2 and lower COLL1A1 expression compared to the respective TCP0. Downregulation of the COL1A1 gene was attributed to the fact that the protein deposition was probably completed at this time, as supported by the immunofluorescent images (**Figure 5D**). In addition, upregulation of BMP2 could indicate that a more active matrix remodeling was ongoing on TCP40 scaffolds.

Finally, we assessed the expression of Osteocalcin (OCN), a non-collagenous protein of the bone extracellular matrix secreted by osteoblasts at late stages of maturation[67]. At both time points, OCN gene was not expressed. In addition, OCN was neither observed in immunofluorescent images (**Figure S11C**). The absence of OCN together with the constant RUNX2 expression over time suggested that the cells did not yet reach the final maturation stage after 28 days [68-71]. The slow exposition of CaP may have delayed the tissue maturation.

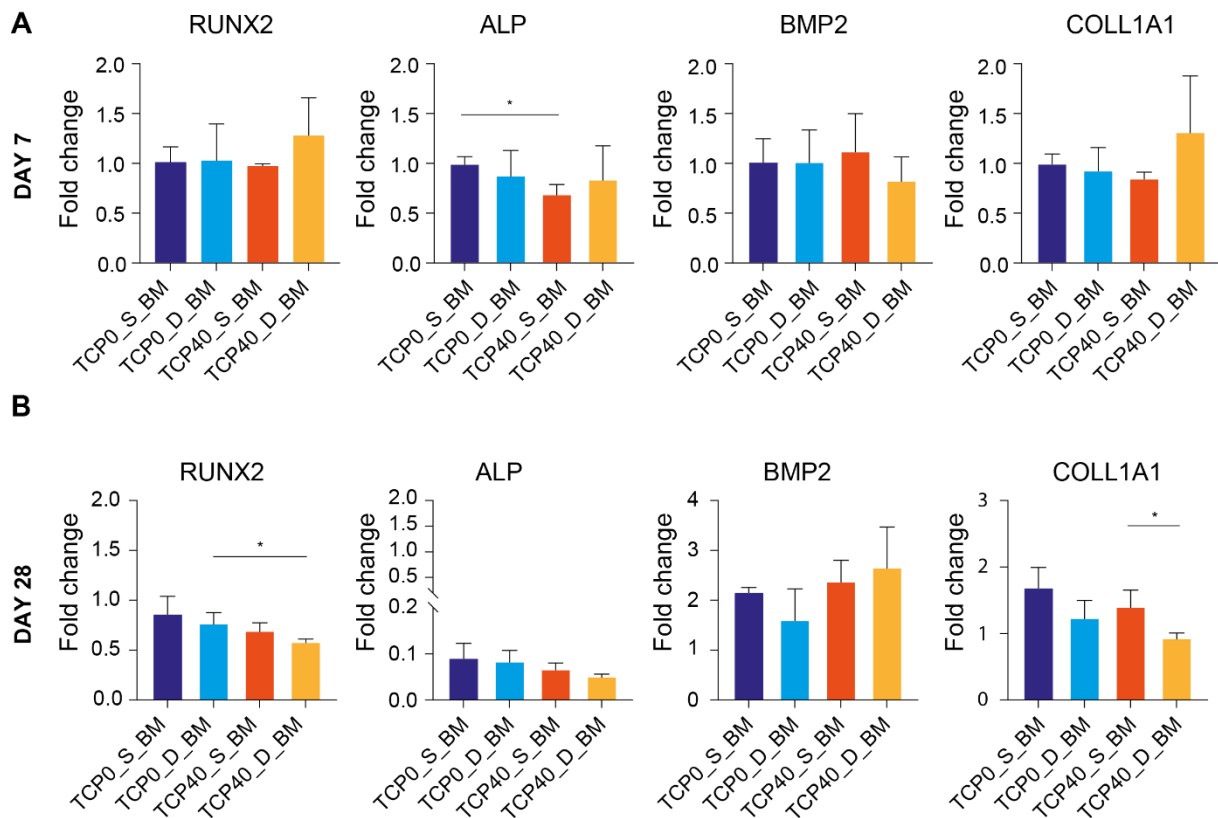


Figure 7. Gene expression of hMSCs cultured in BM on TCP40 and TCP0 scaffolds for 7 (A) or 28 days (B), n=3.

Taken together, the addition of a high amount of TCP enhanced the deposition of mineralized matrix, whereas the effect on the gene expression and matrix composition was limited. In comparison, our findings align with prior studies that demonstrated that adding ceramic particles to a polymeric matrix did not significantly enhance the osteogenic potential of scaffolds prepared with melt-extrusion additive manufacturing. For example, addition of 20% (w/w) hydroxyapatite to PCL/poly(lactic-co-glycolic) acid or 30% (w/w) TCP to PCL scaffolds had limited effect on the expression of osteogenic markers of hMSCs in vitro, compared to pristine polymer scaffolds [72, 73]. Similarly, poly(ethylene oxide terephthalate)/poly(butylene terephthalate) scaffolds containing 45% w/w HA or 50% w/w TCP did not enhance osteogenic potential of the constructs in vitro [61, 74]. In these studies, similarly to the considerations made in our work, the results were attributed to the low availability of CaP particles to the cells. We believe that degradation of the superficial polymer layer would enhance particle exposure, which may improve the osteogenic potential of these composite scaffolds. To this end, surface modifications have been proposed, such as alkaline etching with NaOH [50, 75] or coatings with ceramic materials [76, 77].

Regarding the effect of the S and D scaffolds, the results of biochemical assays and mineralization confirmed the initial hypothesis that different pore geometries influence

hMSCs differentiation. Notably, the D-shaped scaffolds exhibited increasing trends in ALP activity and collagen production, while the S scaffold induced higher mineralization after 28 days. Prior research has explored the impact of pore shape on cell differentiation. For instance, *in vitro*, higher ALP activity and osteogenic gene expression was found in PCL or titanium scaffolds with rhomboidal or triangular pores, compared to the more traditionally used woodpile geometry [27, 78]. Other studies have shown that *in vivo* diamond shaped scaffolds exhibited greater bone formation than the square, circular or triangular pore [79, 80]. In our study, the overall effect of the shapes appeared limited, namely with the D-shaped scaffolds having an effect on early osteogenic differentiation (i.e. ALP activity increase) and the S-shaped scaffolds on late osteogenic differentiation (i.e. mineralization increase), which we attributed to the static culture conditions. We identified this as a potential limitation of the study, which in future studies could be improved. In fact, Rubert et al. demonstrated that dynamically compressing scaffolds with square or circular pores induced a stronger effect of the geometry on the differentiation of seeded hMSCs. This result was ascribed to mechanical straining of cells and higher nutrient transport under dynamic stimulation [81]. Moreover, static cultures resemble the physiological environment *in vivo* less than the dynamic conditions. For example, Hutmacher et al. noted that while *in vitro* triangular-shaped pores supported cell differentiation, *in vivo* the bone formation was low, which was attributed to the complexity of the *in vivo* system, where growth factors arrive at the wound site at different stages of healing [82]. Consequently, we believe that future *in vivo* studies are needed to better conclude on the impact of S and D scaffolds on bone formation.

Conclusion

In this study, scaffolds with a star- (S) or diamond- (D) like pore shape, were fabricated via melt-extrusion based manufacturing. The scaffolds comprised of either pristine PCL (TCP0) or PCL with high amounts of β -TCP (40% w/w, TCP40). The morphological evaluation of the scaffolds revealed a similar total porosity, but a higher average pore volume in the D scaffolds. The D scaffolds displayed higher mechanical properties than the S ones, and the addition of TCP acted as reinforcing filler when the different chemical scaffold composition were compared. Moreover, after 28 days, TCP40 samples favored mineral deposition in SBF solutions, indicating bioactive properties. Thereafter, we evaluated the effect of pore shape and material on osteogenic differentiation of hMSCs. At early time points, expression of RUNX2 implied the commitment of the cells towards osteogenic phenotypes in all scaffolds. The ALP activity and collagen I deposition displayed increasing trends for the D-shaped scaffolds. The gene expression indicated that osteogenic genes were upregulated, and we found that TCP40 scaffolds supported the deposition of mineralized matrix. Notably, a higher deposition of Ca pockets was observed

in TCP40_S scaffolds than TCP40_D, which could be a result of the higher surface area of the S scaffolds. Based on the evaluation of the gene and protein expression, we concluded that the cells did not reach full differentiation after 28 days. Thus, to accelerate the biological effect of scaffolds, a surface etching method could be applied to expose the CaP particles to the cells at earlier time points. Nonetheless, taken together, by a thorough physicochemical and biological characterization, we demonstrated the in vitro osteogenic potential of scaffolds with complex pore architectures, especially those comprised of a composite material. As the effect of physiological parameters such as dynamic mechanical loading were not assessed, in future studies we will aim at further investigating these scaffold architectures in a maxillofacial critical sized bone defect in a porcine model.

Acknowledgments

We would like to kindly acknowledge Corbion for providing the PC12 polymer and Kuros for the β -TCP. Micro-CT investigations were possible due to ERDF/COP 2014-2020, ID P_36_611, MySMIS 107066, INOVABIOMED.

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CHAPTER 3

Supplementary information

PCL/ β -TCP composite scaffolds with advanced geometries promote hMSCs osteogenic differentiation

Sophia Dalfino, Elena Olaret, Marco Piazzoni, Paolo Savadori, Izabela Stancu, Gianluca Tartaglia, Claudia Dolci, Lorenzo Moroni

Dalfino et al., *Tissue Engineering Part A*, 2025, 10.1089/ten.TEA.2024.0030

Material and methods

Table S1. Reagent amounts for 1000 mL of SBF.

Reagent	Amount
NaCl	8.036 g
NaHCO ₃	0.352 g
KCl	0.225 g
K ₂ HPO ₄ · 3H ₂ O	0.230 g
MgCl ₂ · 6H ₂ O	0.311 g
1.0 M HCl	40 mL
CaCl ₂	0.293 g
Na ₂ SO ₄	0.072 g
TRIS	6.063 g
1.0 M HCl	~ 0.2 mL

Table S2. Primer sequences of the genes, used for RT-qPCR experiment: runt-related transcription factor (RUNX2), alkaline phosphatase (ALP), bone morphogenetic protein (BMP2), collagen I alpha I (COL1A1), osteocalcin (OCN), and beta-2-microglobulin (B2M). B2M was used as housekeeping gene.

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
RUNX2	TCAACGATCTGAGATTTGTGGG	GGGGAGGATTTGTGAAGACGG
ALP	ACAAGCACTCCCACTTCATC	TTCAGCTCGTACTGCATGTC
BMP2	ACTACCAGAAACGAGTGGGAA	GCATCTGTTCTCGGAAAACCT
COL1A1	CGGTGGTTTCTTGGTCGGT	GTGCGATGACGTGATCTGTGA
OCN	TGAGAGCCCTCACACTCCTC	CGCCTGGGTCTCTTCACTAC
B2M	ACAAAGTCACATGGTTCACA	GACTTGTCTTTAGCAAGGA

Results and discussion

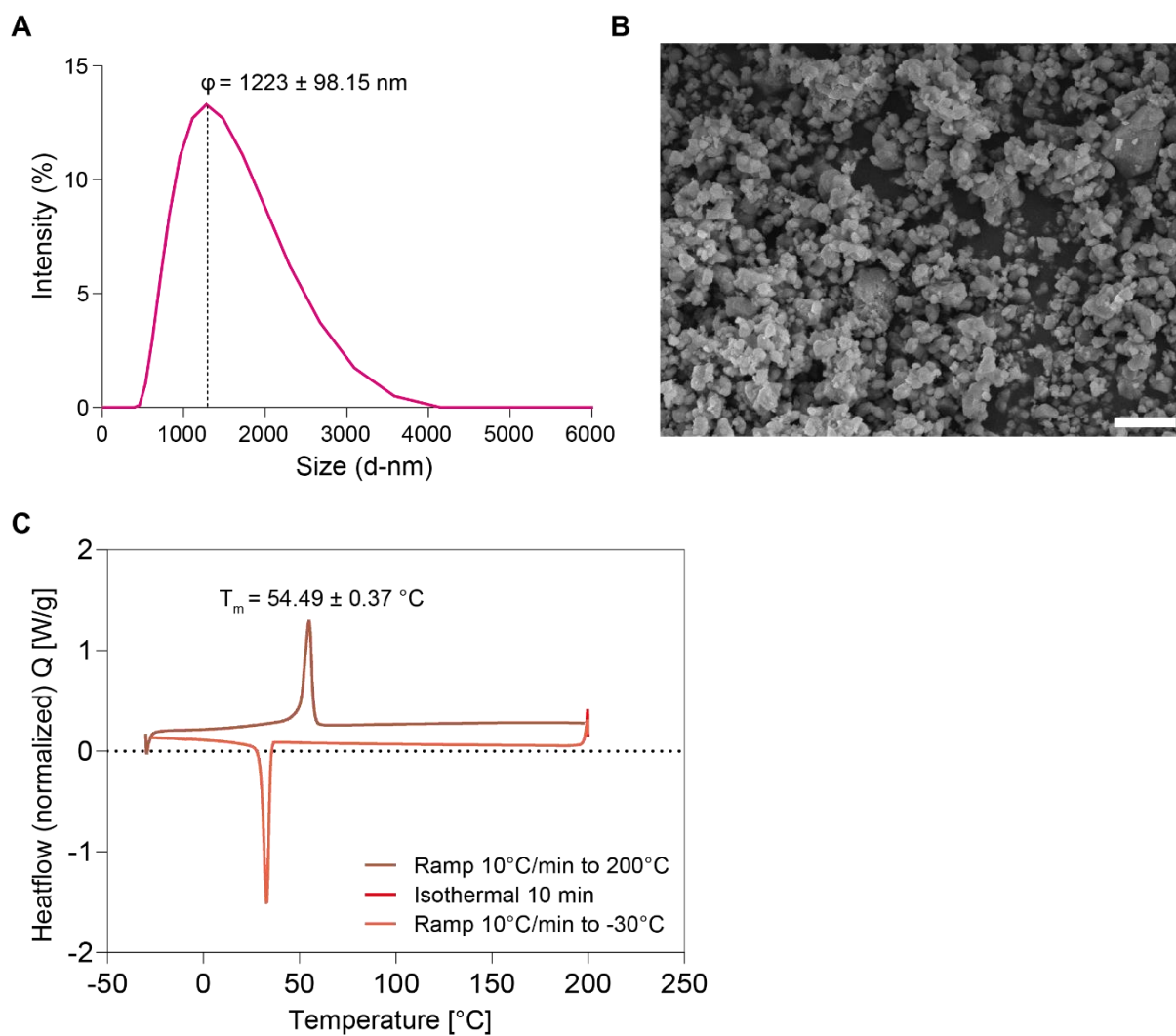


Figure S1. DLS measurements showing the average TCP particle size(A); SEM image of the TCP particles. Scale bar = 5 μm (B). DSC analysis of the TCP40 material, showing the melting temperature (C).

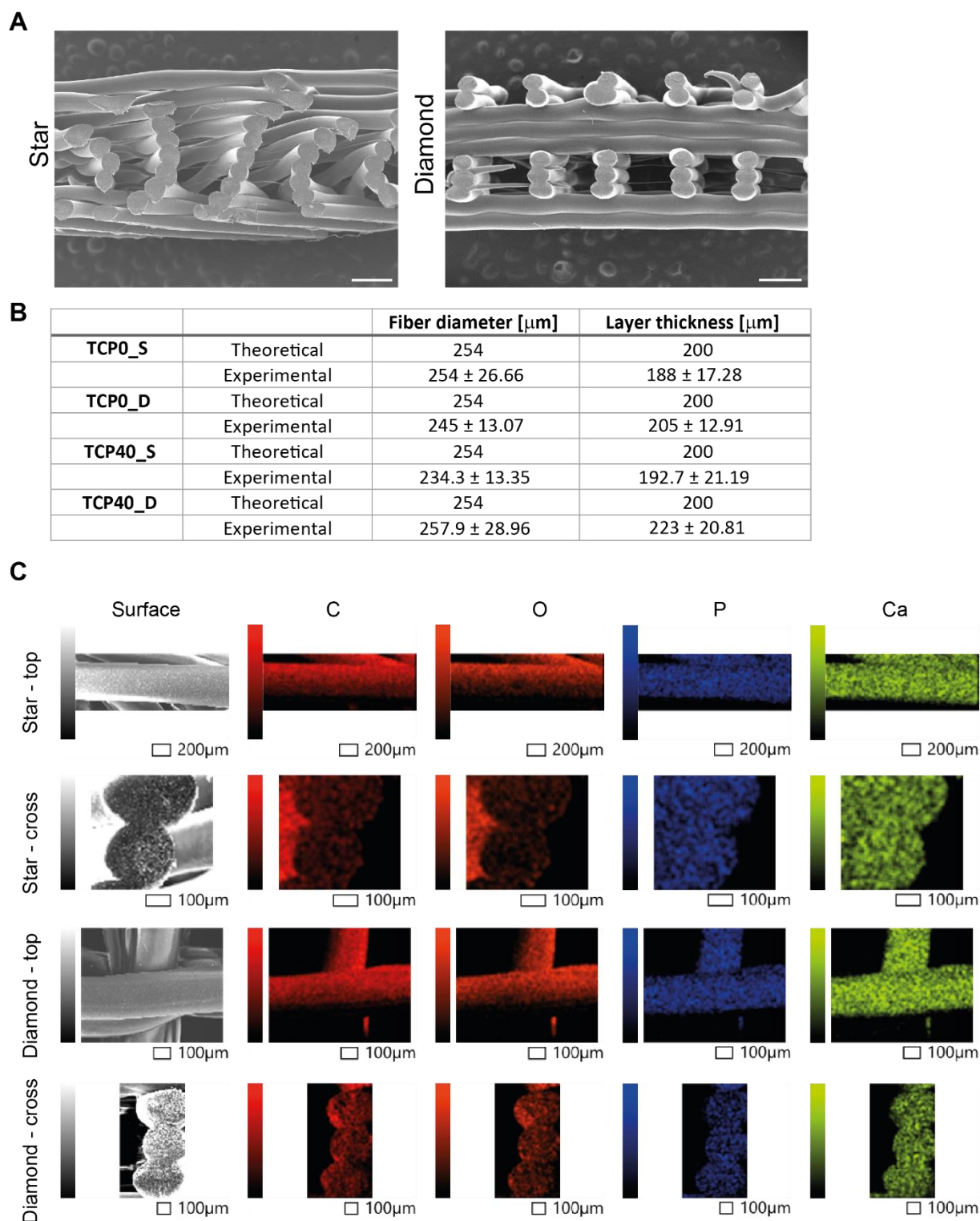


Figure S2. SEM images of the cross-section of S and D TCP40 scaffolds. Scale bar = 500 μm (A). Morphological printing parameters measured with ImageJ for all the printed scaffolds (B). Elemental map showing the element distribution of TCP40 S and D scaffolds on the surface and in the fiber cross-section (C).

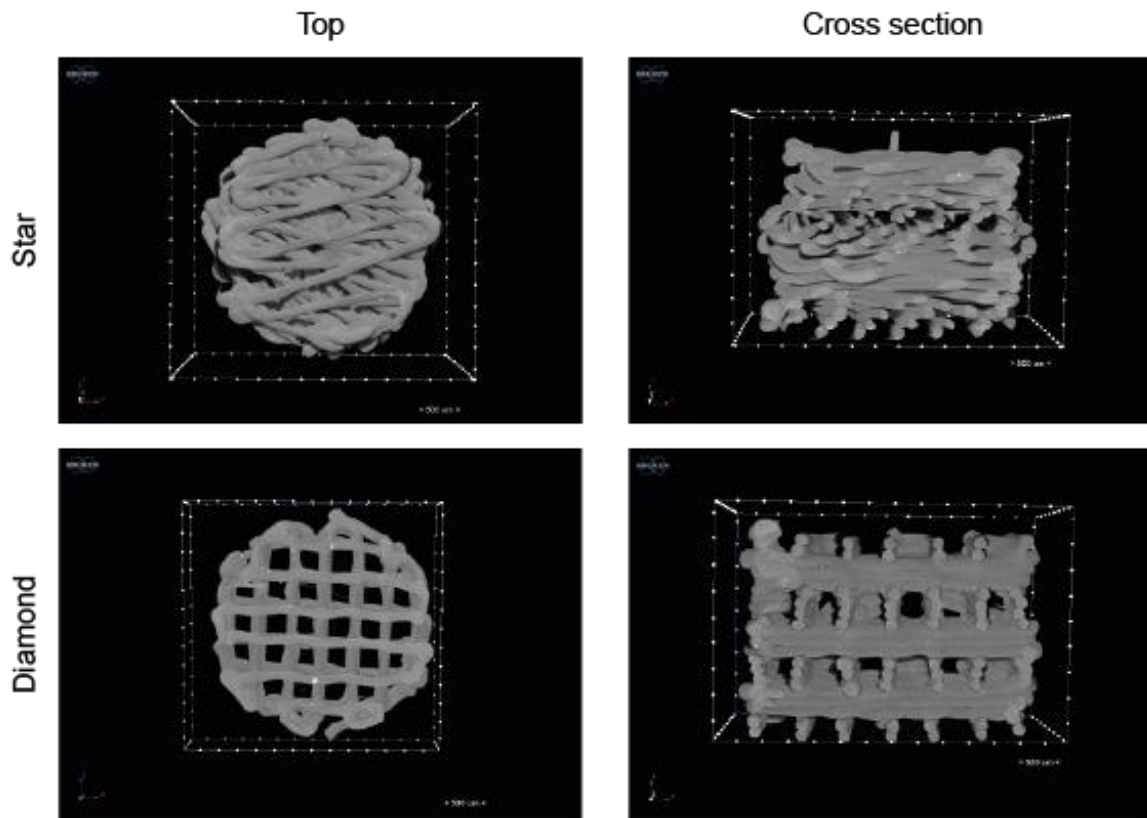
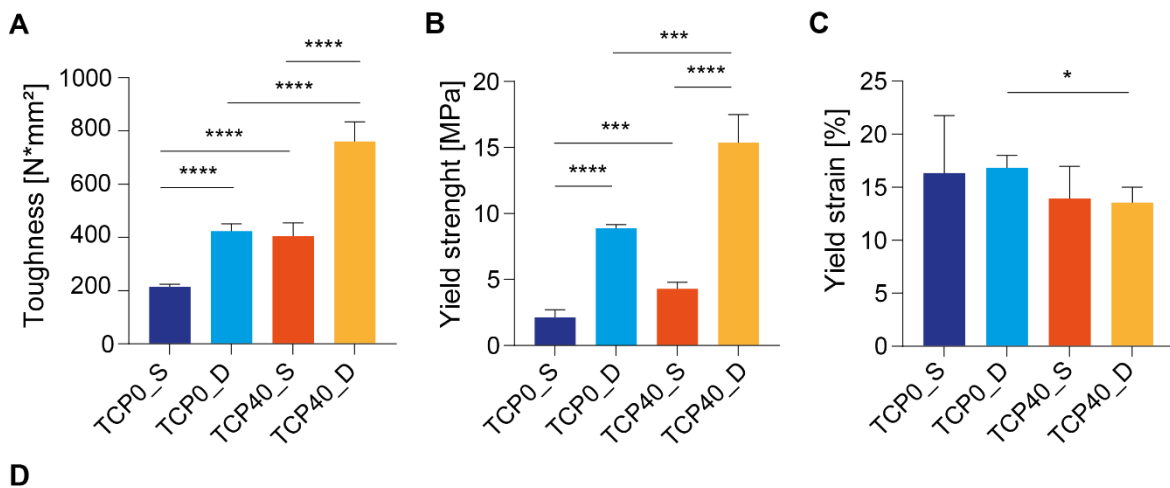


Figure S3. MicroCT images of the top and cross-section of S and D TCP40 scaffolds.



D

	Compressive modulus [MPa]	Toughness [N*mm²]	Yield strength [MPa]	Yield strain [%]
TCP0_S	17.96 ± 2.3	214.9 ± 10.1	2.13 ± 0.57	16.32 ± 5.44
TCP0_D	103.2 ± 8.1	423.7 ± 27.7	8.88 ± 0.16	16.83 ± 1.18
TCP40_S	42.59 ± 6.7	404.8 ± 49.6	4.28 ± 0.5	13.92 ± 3.04
TCP40_D	277.2 ± 16.3	760.3 ± 74.45	15.36 ± 1.12	13.54 ± 1.46

Figure S4. Mechanical characterization of the TCP0 and TCP40 star (S) or diamond (D) scaffolds: toughness (A), yield strength (B), and yield strain (C). Mechanical parameters for the TCP0 and TCP40 scaffolds, with S and D shape: compressive modulus, toughness, yield strength and yield strain are reported (D).

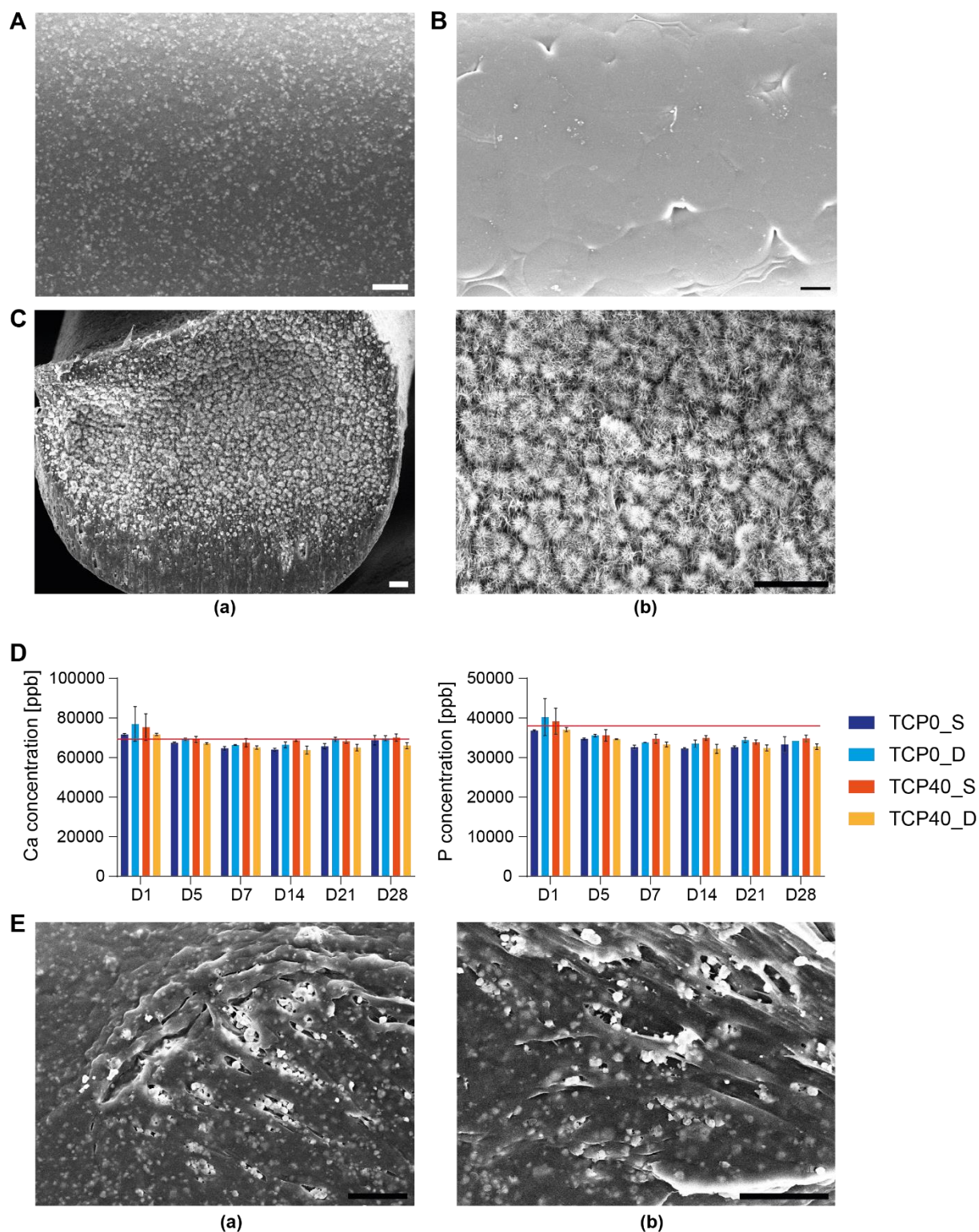


Figure S5. Representative SEM image of TCP40_D after 7 days in SBF. Scale bar = 10 μm (A). Representative SEM image of TCP0_S after 28 days in SBF. Scale bar = 20 μm (B). Representative images of TCP40_S fiber cross section after 7 days in SBF, at different magnifications ((a) and (b)). Scale bar = 10 μm (C). Ca and P concentrations in cell culture medium measured with ICP-MS at different time points. The red line refers to the concentration of the blank medium without scaffolds (D). Representative SEM image of TCP40_D scaffolds after 28 days in culture medium, at different magnifications ((a) and (b)). Scale bar = 10 μm (E).

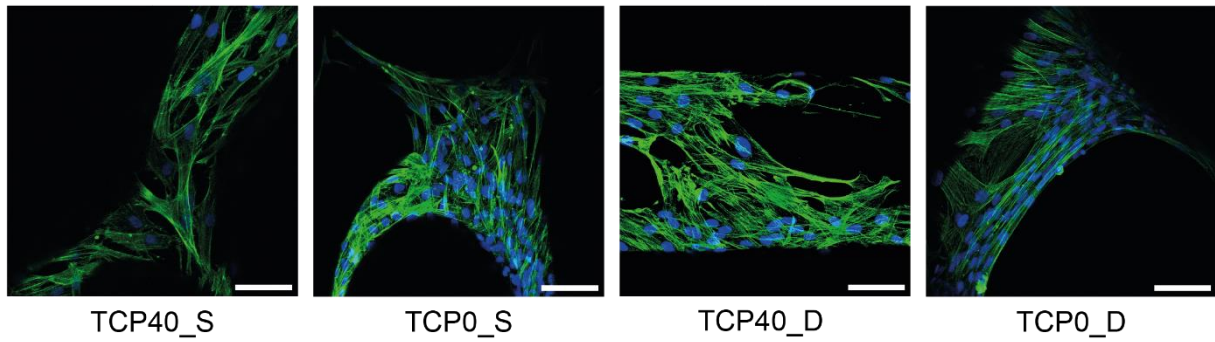
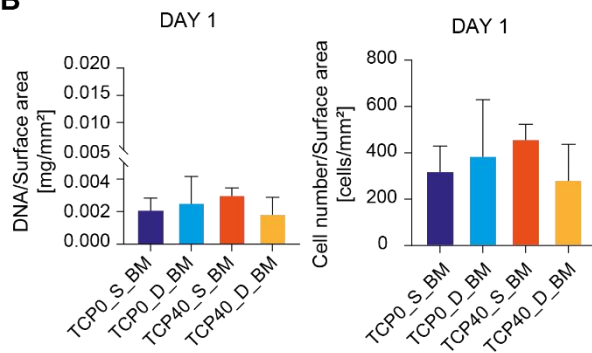
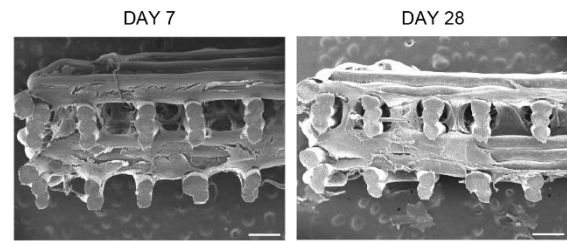
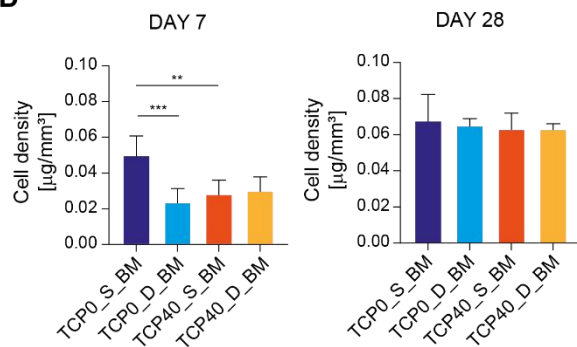
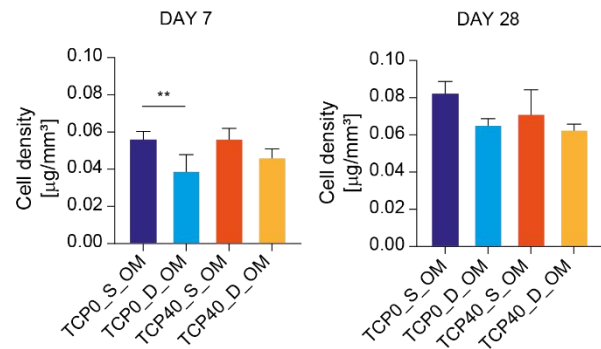
A**B****C****D****E**

Figure S6. Representative immunofluorescent confocal images at 24h from the seeding, showing in blue the nuclei (blue) and F-actin (green). Scale bar = 200 μ m (A). DNA content and cell number normalized against the available surface area at day 1 in BM (B). Representative SEM images of TCP40_D scaffolds at day 7 and 28 showing the presence of cells in the pores. Scale bar = 500 μ m (C). Cell density at day 7 and 28 in BM (D) and OM (E).

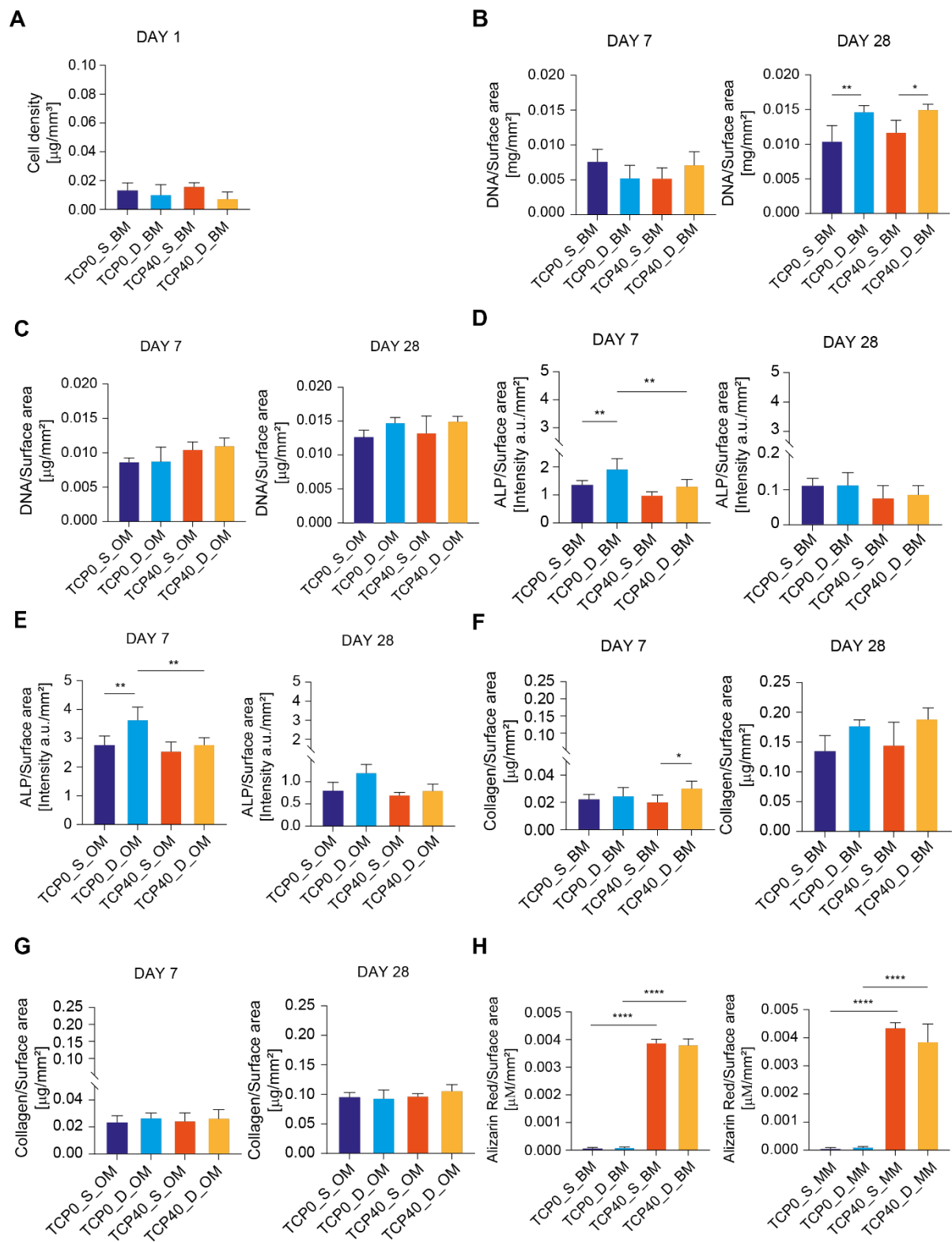


Figure S7. Cell density at day 1 in BM (A). Normalization against available surface area: DNA at day 7 and day 28 in BM (B) and OM (C). ALP activity at day 7 and day 28 in BM (D) and OM (E). Collagen production at day 7 and day 28 in BM (F) and OM (G). Alizarin Red normalized against the available surface area in BM and MM (H).

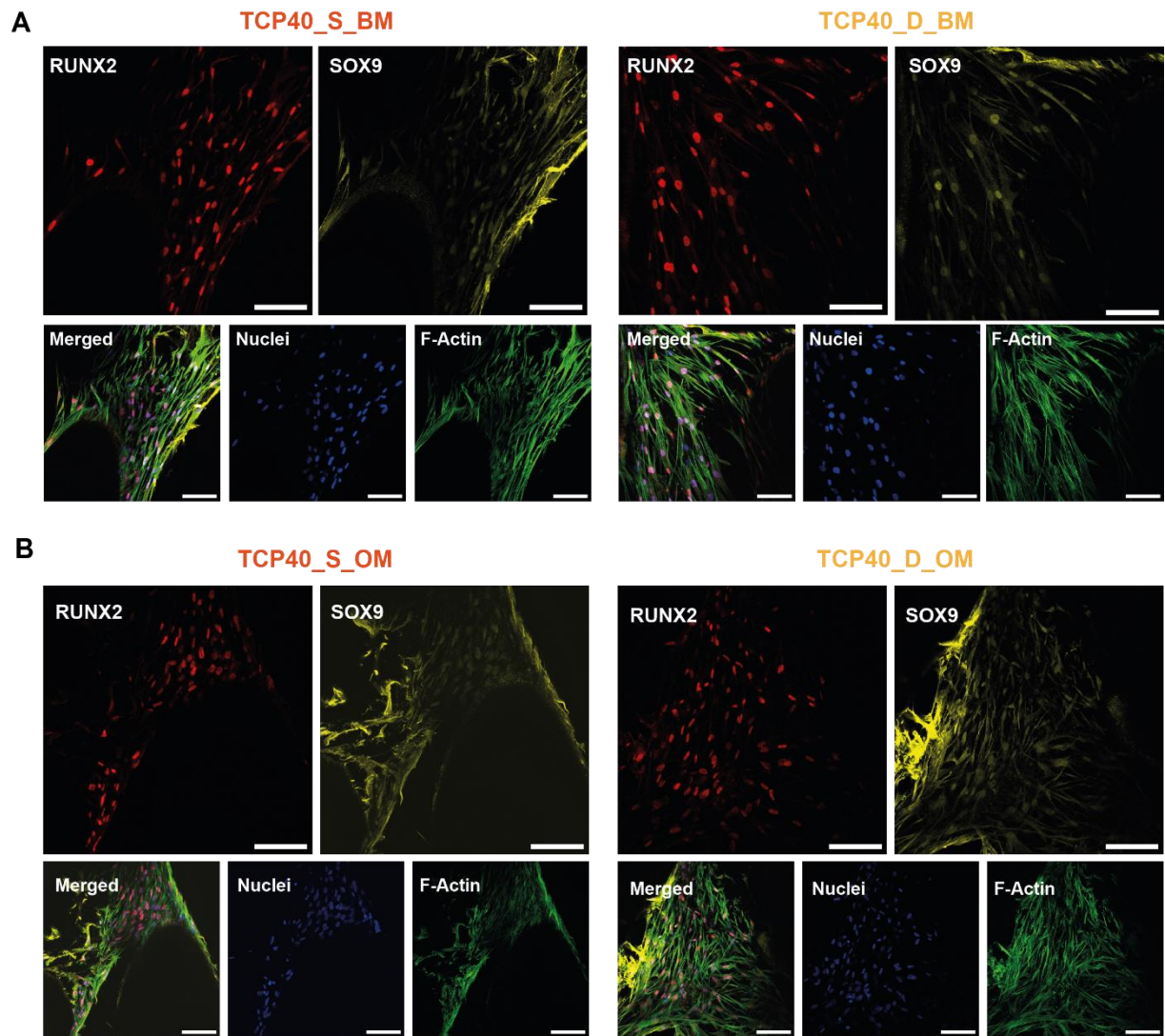


Figure S8. Representative confocal images of TCP40_S and TCP40_D scaffolds at day 7 in BM (A) or OM (B). The samples were stained for RUNX2 (Red), SOX9 (yellow), Nuclei (blue), and F-actin (green). Scale bar = 200 μ m.

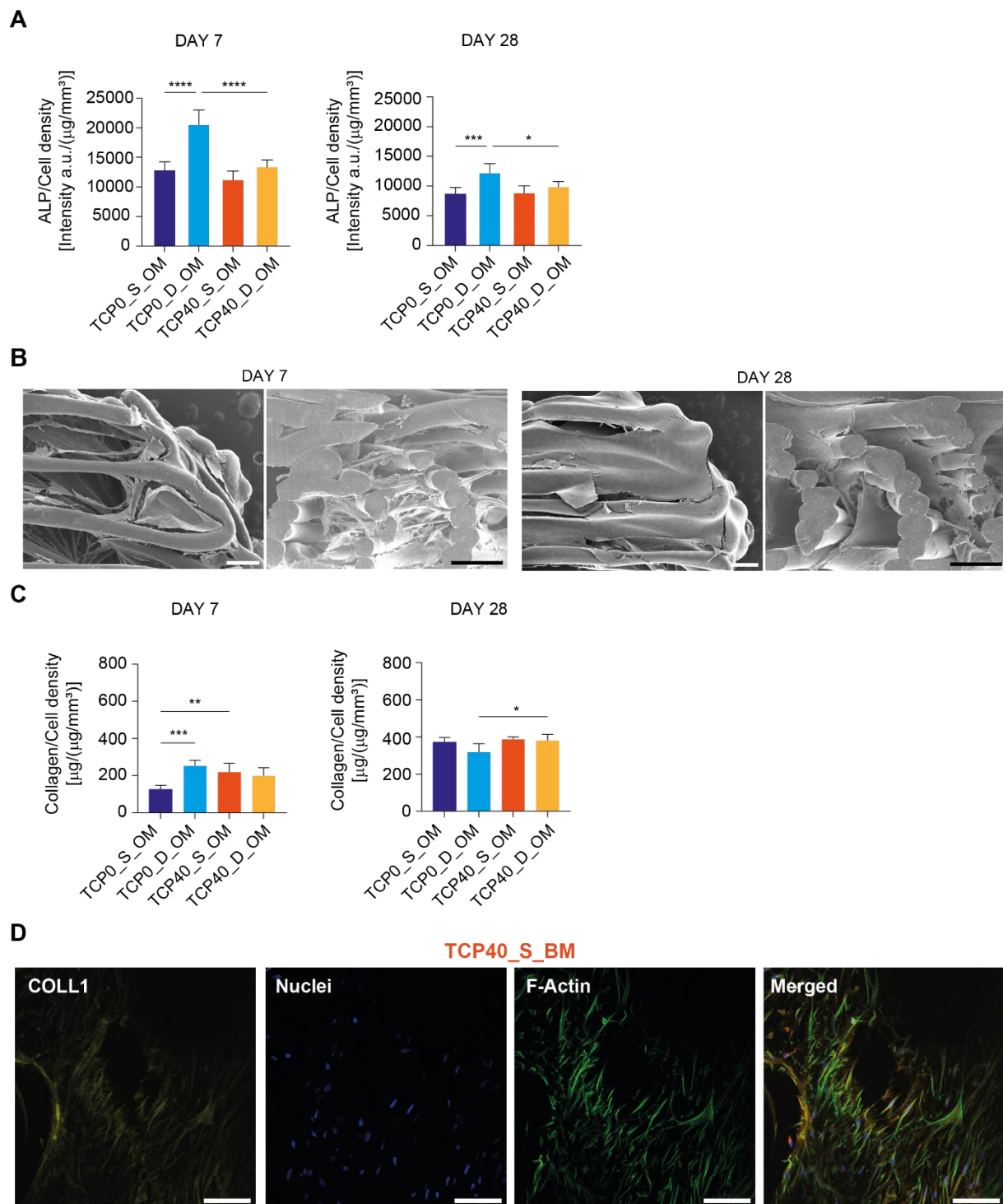


Figure S9. Normalized ALP activity at day 7 and 28 in OM (A). Representative SEM image of ECM production in TCP40_S scaffolds (top and cross section) at day 7 and 28 in BM. Scale bar = 500 μm (B). Normalized collagen production at day 7 and 28 in OM (C). Representative confocal images of TCP40_S scaffolds in BM, staining Collagen I (yellow), nuclei (blue) and F-actin (green). Scale bar = 200 μm (D).

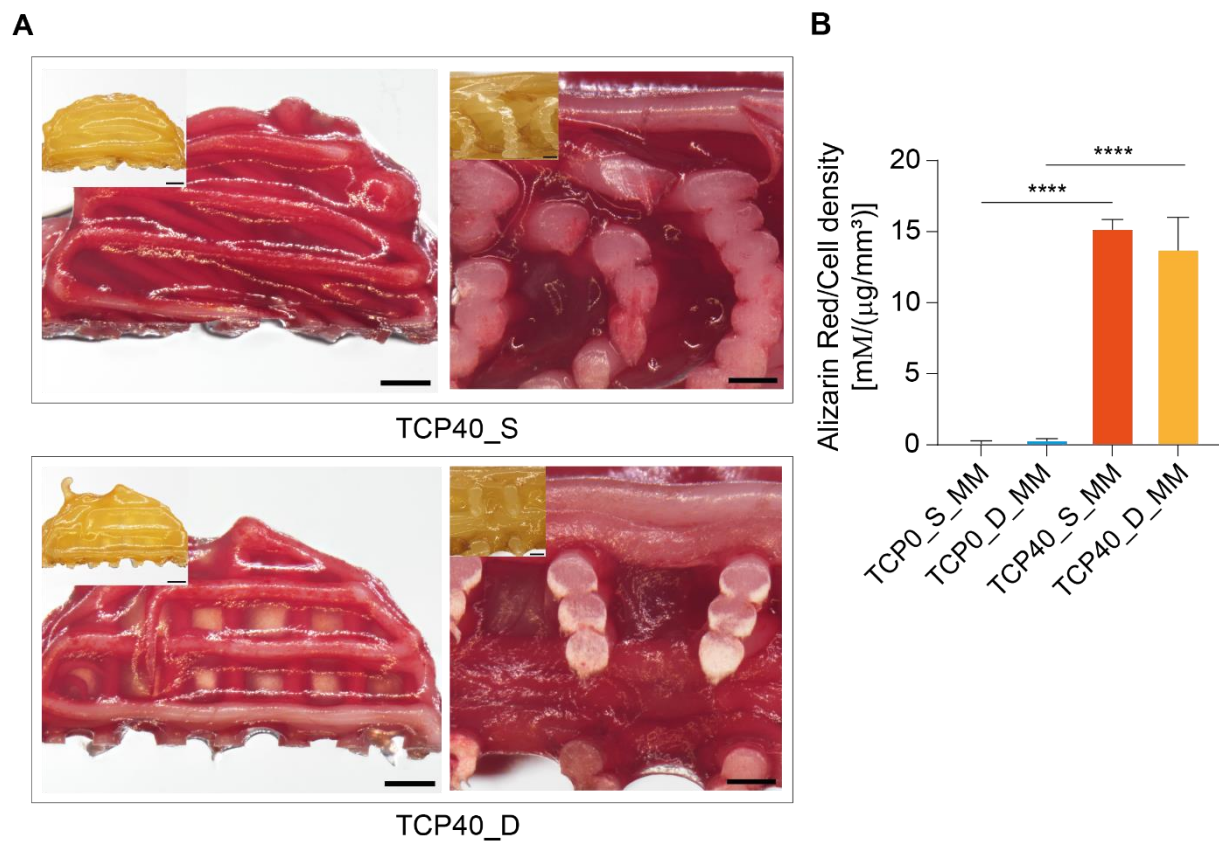


Figure S10. Stereomicroscope images of ARS at day 28 in MM, for TCP40_S and TCP40_D samples, from the top and of the cross section. In the up-left corner, the corresponding TCP0 scaffolds. Scale bar = 200 μm (A). ARS quantification at day 28 in MM (B).

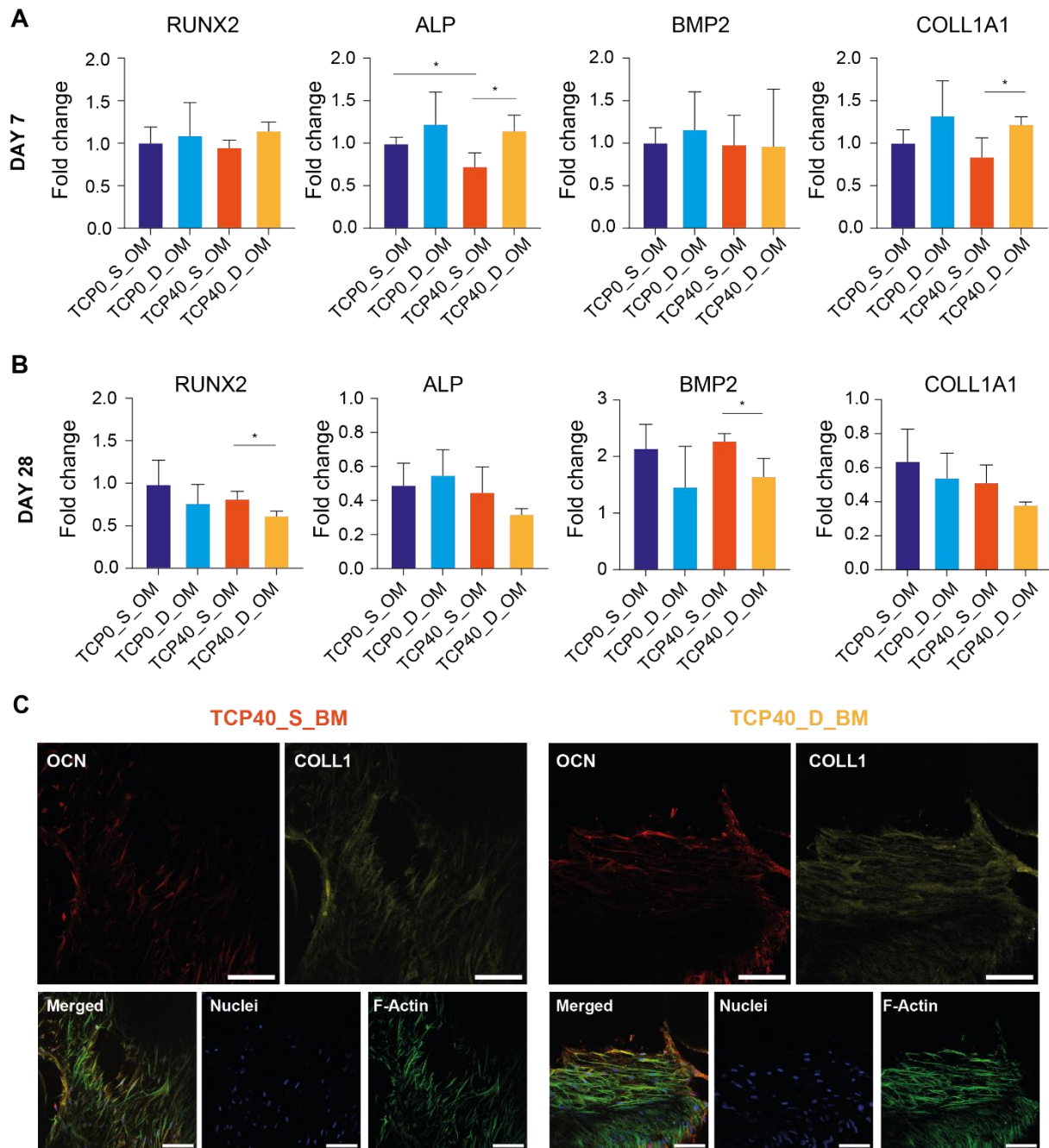


Figure S11. Gene expression of hMSCs cultured in OM on TCP40 and TCP0 scaffolds for 7 (A) or 28 days (B). Representative confocal images of TCP40_S and TCP40_D in BM after 28 days of culture. Samples were stained for OCN (red), COLL 1 (yellow), Nuclei (blue), F-actin (green). Scale bar = 200 μ m (C).

CHAPTER 4

Additive manufacturing and in vitro characterization of scaffolds consisting of PCL/P₂O₅-free bioactive glasses composites with angiogenic and osteogenic potential

Martyna Nikody, Sophia Dalfino*, Pamela Habibovic, Lizette Morejón, José A. Delgado, Gianluca Martino Tartaglia, Claudia Dolci, Elizabeth R. Balmayor, Lorenzo Moroni*

Submitted and under revision at Biomaterials (Elsevier)

**These authors contributed equally to this manuscript*

Abstract

The development of scaffolds with osteogenic and angiogenic properties represents an attractive strategy for regenerating large bone defects. This study describes the fabrication and characterization of bioactive glass-based composite scaffolds for bone regeneration applications. Scaffolds comprising polycaprolactone (PCL) and two different bioactive glasses (BGs), derived from white sand (BG-WS) and yellow sand (BG-YS), at an 80:20 wt% ratio were fabricated with a star-shaped pore geometry. Chemical characterization confirmed the presence of characteristic groups from PCL and BGs. The incorporation of BGs led to increased compressive moduli in the composites compared to PCL scaffolds only. Dissolution products, particularly from PCL/BG-YS, promoted enhanced vascular pattern formation of human umbilical endothelial vein cells (HUVECs) co-cultured indirectly with human mesenchymal stromal cells (hMSCs). Direct culture of hMSCs on PCL/BG-WS and PCL/BG-YS composite scaffolds resulted in greater VEGFA production compared to PCL scaffolds alone. The expression of the early osteogenic marker *RUNX2* was upregulated on day 7 in response to both composite scaffolds, while *ALPL* expression increased only with the PCL/BG-WS scaffolds on day 28. Additionally, late osteogenic gene markers (*COL1A1* and *OCN*) were both upregulated on day 28 in PCL/BG-WS scaffolds. Lastly, the measurement of analytes secreted by hMSCs showed higher secretion levels on day 7 than 28, with PCL scaffolds yielding higher concentrations of IL-10, TNF- α , and CCL2 than composite scaffolds. The findings of this study indicate that combining naturally-derived P₂O₅-free BGs with PCL to fabricate 3D composite scaffolds enhances osteogenic and angiogenic properties of the final constructs, emphasizing their potential in bone regeneration.

Keywords

Bioactive glasses, Composite scaffolds, Osteogenesis, Angiogenesis, Immunomodulation

Introduction

The continuing ageing of the population together with an increasing occurrence of diseases affecting the skeletal system stimulates the research and development of new implantable materials [1]. Nevertheless, bone grafting using native tissue remains the gold standard treatment in the clinical setting [2]. However, autologous or allogenic grafts present several drawbacks, including limited tissue availability, surgical invasiveness, and donor-site morbidity [3].

The field of bone tissue engineering (BTE) aims to address these limitations by developing porous scaffolds, that can support, guide, and enhance the formation of new bone tissue. To effectively mimic the native bone, scaffolds are carefully designed to possess suitable chemical, morphological, and mechanical properties [4]. Melt extrusion-based additive manufacturing (AM), based on a layer-by-layer deposition process, has emerged as an effective method to fabricate porous scaffolds with precise control over their architecture [4]. This technique accommodates a wide range of materials, including biocompatible and biodegradable thermoplastics such as polycaprolactone (PCL) and polylactic acid (PLA), which provide favorable mechanical properties and support long-term tissue growth [4]. These polymers can be combined with inorganic materials to better recapitulate the composition of native bone and enhance osteogenic activity [5].

However, promoting angiogenesis within engineered constructs remains a challenge that needs to be addressed in order to prevent implant failure [6]. To this end, research has focused on the addition of pro-angiogenic cues promoting vascularization into the composites such as bioactive molecules [7], inorganic ions [8], or bioactive glasses (BGs) [9,10].

BGs are a class of amorphous ceramic materials that have been used in the field of bone regeneration for their pro-angiogenic properties [11]. BGs are generally categorized into silicate-based, phosphate-based, and borate-based classes [12], with properties tunable by varying their chemical composition or by adding or removing functional elements like phosphorus, copper, or strontium [13]. The majority of the mainstream BGs contain P_2O_5 in their composition as a source of phosphate groups that participate in hydroxyapatite formation [14,15]. However, studies have shown that the presence of phosphorus can increase the susceptibility of the glass network to hydrolysis. This effect is associated with the formation of P-O-P and P-O-Si bonds, which are more easily cleaved in aqueous environment, resulting in accelerated ion release, uncontrolled dissolution, and faster loss of mechanical integrity [16–18]. A more rapid dissolution can compromise mechanical stability and shorten the time of biological effectiveness, representing undesirable features for bone tissue engineering applications that require a gradual degradation rate. In contrast, BG formulations without P_2O_5 exhibit enhanced chemical durability and more predictable dissolution behavior due to absence of easily hydrolyzed

P-O-P and P-O-Si bonds, contributing to a more stable and sustained bioactive response [16–18]. Importantly, several studies have shown that, despite the absence of P_2O_5 , such BGs are still capable of apatite formation under physiological conditions and can stimulate biological processes involved in bone regeneration through the controlled delivery of Si, Ca, as well as trace element ions [19–21]. These properties make P_2O_5 -free glasses particularly suitable for applications requiring controlled degradation rates and sustained release of bioactive ions, such as bone defect repair and regeneration [18,21,22].

Specifically, novel P_2O_5 -free BGs from the SiO_2 -CaO- Na_2O system, synthesized from calcite minerals and silica sands, extracted from natural deposits, have demonstrated promising osteogenic and angiogenic capabilities [19,20]. Among these BG formulations, BG-WS and BG-YS exhibited the most promising biological performance [19]. BG-WS and BG-YS were synthesized from natural raw materials, including calcite and silica sands extracted at different depths of the sand deposit, which influenced both the colour and composition of the silica sands [23]. Specifically, BG-WS was obtained using white silica sand collected from shallower layers of the deposit, while BG-YS originated from yellow silica sand extracted from deeper layers. These depth-related differences translated into distinct trace element profiles: BG-WS contained a higher amount of titanium (Ti) ions, while BG-YS had more aluminum (Al) and iron (Fe) ions [23]. Both BG formulations have influenced osteogenic and angiogenic properties, with BG-YS being more potent at influencing osteogenic and angiogenic properties on a gene level, while BG-WS exerted a pronounced effect on vascularization in a CAM assay [19].

In this study, PCL was chosen as the polymeric component of the composite due to its biocompatibility, biodegradability, relatively high mechanical properties, ease of processability, and support for *in vitro* and *in vivo* tissue growth [24]. Composite scaffolds composed of PCL blended with BG-WS or BG-YS (80:20 wt%) were fabricated using melt-extrusion AM, characterized and evaluated *in vitro* for their osteogenic, angiogenic, as well as immunomodulatory properties using clinically relevant human mesenchymal stromal cells (hMSCs) and human umbilical vein endothelial cells (hUVECs).

Materials and methods

Raw materials

Medical grade PCL (PURASORB® PC08, Mw = 51 kDa) was provided by Corbion (Amsterdam, The Netherlands). All chemicals were bought from Merck KGaA (Darmstadt, Germany) unless stated otherwise.

BGs used in this study were previously synthesized and characterized [23] and their osteogenic as well as angiogenic properties were reported [19,20]. BGs were composed of silica sands and calcite extracted from natural resources. Depending on the depth of the deposit from which the sand was extracted, its corresponding colour varied: white sand

(WS, quartzose sand, depth up to 0.7 m), and yellow sand (YS, quartzose sand, depth range 1.25 – 1.85 m). The content of potentially harmful levels present in both silica sands and calcite was below the limits established by the Standard Specification for Glass and Glass-Ceramics Biomaterials for Implantation (ASTM F1538), indicating that they could be used directly for clinical applications without the need for additional purification steps [23]. Starting from either WS or YS type of silica, corresponding glasses have been synthesized and termed BG-WS and BG-YS. The raw materials were combined with sodium carbonate anhydrous and subjected to a multi-step thermal treatment: the temperature was first increased to 650 °C at a rate of 2 K/min, then to 950 °C at 1 K/min, and finally to 1450 °C at 5K/min. The melt was maintained at 1450 °C for 3 h before being rapidly quenched onto stainless steel plates preheated to 400 °C. The resulting glasses were annealed at 450 °C for 2 h and then gradually cooled down [23]. The BG-WS and BG-YS powders were obtained as larger pellets (mm sized) and were treated with ball milling for 90 min to reduce the particle size to facilitate powder processability. To assess the obtained particle size, the powders were gold-coated (Cressington sputter coater 108 Auto) and imaged with scanning electron microscopy (SEM, Jeol JSM-IT200 InTouchScope, Tokyo, Japan), with a voltage of 10 kV and a working distance of 10 mm. Finally, the chemical composition of the powders was validated via an energy-dispersed X-ray spectroscopy (EDX) analysis.

Preparation and characterization of PCL/BG composites

Starting from BG-WS and BG-YS powders, two composite materials were produced, PCL/BG-WS or PCL/BG-YS, following a previously optimized blending method [25]. Briefly, BG-WS or BG-YS powders were added to PCL dissolved in tetrahydrofuran, in a PCL:BG ratio 80:20 w/w. A 20% w/v glass content was selected as the highest that allowed reliable and reproducible printing process, without clogging or loss of printing fidelity. Once the powders were homogeneously dispersed in the solution through mechanical stirring, the composites were precipitated in ethanol and dried in the vacuum oven at 30 °C overnight to remove excess of solvent [25]. The chemical composition of the composites was validated via X-Ray Diffraction (XRD, D2 phaser, Bruker, Massachusetts, USA) and Fourier-Transform Infrared Spectroscopy (FTIR, Nicolet iS50, Thermo Scientific, Massachusetts, USA). XRD analysis was performed in the range $7^{\circ} \leq 2\theta \leq 60^{\circ}$, and increments of 0.02° , while the FTIR was conducted in the $4000\text{--}400\text{ cm}^{-1}$ range, running 16 background scans and 32 scans. Raw BG powders and PCL were included in the analysis as controls.

Scaffold fabrication and alkaline surface etching

PCL only and composite scaffolds, PCL/BG-WS and PCL/BG-YS, were printed with an in-house developed extrusion-based printer [26]. The materials were added to the reservoir and heated at $T=95\text{ }^{\circ}\text{C}$ and $T=120\text{ }^{\circ}\text{C}$ for PCL and composites, respectively. Then, the

melted materials were extruded through a 22G nozzle, with a pressure of 3 bar, and a 7.5 rpm screw speed. The extruded filament was deposited at a 3 mm/s translational speed. A star-shaped pore geometry was generated with a GESIM Bioscaffolder software (Radeberg, Germany, version 3.0), setting a rotation of 15 degrees of consecutive printing layers. The geometry was designed by setting a strand distance and a layer thickness of 0.9 mm and 0.33 mm, respectively. Cylindrical scaffolds with 6 mm diameter and 4 mm height were printed for mechanical testing, while for cell culture and degradation experiments, 6 mm diameter and 2 mm height samples were fabricated. Subsequently, the scaffolds were pre-wetted in 70% v/v ethanol for 30 min and then treated for 2 h in a 5M NaOH solution at 37°C, to degrade the superficial layer of polymer and expose the BG particles to the external environment.

Scaffolds characterization

Morphology

The printed scaffolds were gold-coated with a Cressington sputter coater 108 Auto and imaged with SEM, with a voltage of 10 kV and a working distance of 10 mm. Subsequently, the Fiji Software (version ImageJ2) was used to measure morphological parameters, such as fiber diameter, strand distance, layer thickness, and evaluate the printing fidelity. Moreover, the distribution of the BG particles within the fiber and the efficiency of the NaOH treatment were validated.

Mechanical testing

Mechanical properties of the polymer and composite scaffolds were determined upon compression using a TA ElectroForce system (TA Instruments, Delaware, USA), a 450 N load cell, and controlled with Wint7 software. A $0.04 \text{ mm} \cdot \text{s}^{-1}$ strain rate up to 50% compression was applied. Three replicates of PCL, PCL/BG-WS and PCL/BG-YS were tested. The stress-strain curves were derived by dividing the measured load against the net fiber area, calculated as described in previous research works [25,27]. From the stress-strain curves, the compressive moduli of the constructs were calculated in a 5-15% strain range in the linear region.

Degradation, ion release and pH measurements

The degradation of the scaffolds was assessed using three samples of each type. In order to simulate physiological conditions, the samples were incubated in phosphate buffer saline (PBS) containing a physiological concentration of the enzyme lipase (Lip), 102 U/l [28,29]. Prior to the experiment, the scaffolds were weighted and subsequently placed in a 24-well plate containing 2 ml of either PBS or PBS supplemented with lipase (PBS+Lip). The samples were incubated at 37°C for 28 days, with the buffer being replaced every 2-

3 days, and the medium was stored for further analysis. At different time points the scaffolds were collected, dried on absorbent paper from the excess of buffer and weighted. The weight loss percentage was calculated according to the formula (1):

$$\text{Weight loss [\%]} = \left| \frac{W_f - W_i}{W_i} \right| \times 100 \quad (1)$$

Where:

W_i = initial weight

W_f = final weight.

The buffer collected at each degradation time point was analyzed to measure the concentration of biologically relevant ions, Si, Ca, Ti, Fe, and Al. The cumulative release of these ions was quantified by inductively coupled plasma mass spectrometry (ICP-MS, iCAP Q, Thermo Scientific, Massachusetts, USA) in standard mode. Samples were diluted 20 times in 1% v/v nitric acid solution containing 20 ppb scandium as internal standard. For quantification, standard curves in the range of 0.3 - 4.8 ppm for Ca and Si and 0.001 - 0.01 ppm for Ti and Fe were used. Al ions were not possible to quantify due to the very low concentration and low quality of the measurements. To assess the potential effect of ion release on pH changes, scaffolds were immersed in basic cell culture medium and placed in a cell culture incubator (5% CO₂, 37°C). The medium was collected daily and its pH measured, until stabilization around physiological values.

***In vitro* studies**

After washing the scaffolds in water, they were disinfected by immersion in 70% ethanol for 30 minutes, followed by washing steps. Finally, scaffolds were incubated in a basic culture medium composed of α -MEM (Thermo Fisher, Landsmeer, The Netherlands) supplemented with 10% Fetal bovine serum (VWR, Amsterdam, The Netherlands), and 0.2 mM ascorbic acid at 37 °C, 5% CO₂ for 3 days, changing the medium every 24 h to neutralize the pH.

Direct 3D cell culture

hMSCs used in this study were isolated from bone marrow aspirates obtained from one donor, who has given written consent. The studies involving human participants were reviewed and approved by the Medical Ethical Committee (METC) of the Maastricht University Medical Center (#15-4-274). hMSCs were cultured in a basic culture medium at 37 °C under 5% CO₂. The culture medium was changed 24 h after seeding and twice a week during expansion. Prior to cell seeding, the scaffolds were incubated in basic culture medium overnight to allow protein adsorption. After that, the medium was discarded, and scaffolds were dried on blotting paper and placed into 24 well plates. hMSCs at passage 5 were seeded at a density of 200,000 cells per scaffold in a basic culture medium containing 10% of Dextran (Pharmacosmos, Holbæk, Denmark). Cell-seeded scaffolds were incubated

for 4 h to facilitate cell attachment, after which 1.5 mL of basic medium was added. Culture on 3D scaffolds was performed in the above-mentioned basic growth medium with the addition of 100 U/mL penicillin +100 mg/mL streptomycin (Thermo Fisher, Landsmeer, The Netherlands) and, in the case of positive osteogenic control, with the addition of 10 nM Dexamethasone. Medium was refreshed every 24 h for the first 3 days of culture and every 2-3 days afterwards. Cell culture supernatant was collected on days 3, 7, and 28 for downstream analysis.

Osteogenic gene expression

Total RNA was isolated from hMSCs cultured on composite scaffolds for 7 and 28 days using TRIZOL (Thermo Fisher, Landsmeer, The Netherlands), chloroform, and isopropanol. The RNA concentration was measured using a Bio-drop μ LITE instrument (Harvard Biosciences, Massachusetts, USA). cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, California, USA) according to the manufacturer's protocol and diluted in RNase-free water 10 times. The qPCR measurement was performed with Bio-Rad Thermal Cycler (CFX96 Touch Real-Time PCR Detection System, Bio-Rad, California, USA) using Sybr green master mix (Bio-Rad, California, USA). The sequences of the used primers are listed in **Table 1**. Gene expression of *RUNX2*, *ALPL*, *COL1A1*, and *BGLAP* (also known as *OCN* for simplification), was normalized to the housekeeping gene *YWHAZ*, and fold gene expression was calculated using the $\Delta\Delta$ Ct method. Regarding *OCN* expression, the calculated $\Delta\Delta$ Ct values on day 7 were n=2 for PCL/BG-YS in basic medium, n=1 for PCL/BG-WS in osteogenic medium, and n=2 for PCL/BG-YS in osteogenic medium.

Table 1. Primer sequences of the analyzed genes.

GENE	SEQUENCES
<i>YWHAZ</i> (Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta)	F: CCTGCATGAAGTCTGTAAGTCTGAG R: GACCTACGGGCTCCTACAACA
<i>RUNX2</i> (Runt-related transcription factor 2)	F: TCAACGATCTGAGATTTGTGGG R: GGGGAGGATTTGTGAAGACGG
<i>ALPL</i> (Alkaline Phosphatase)	F: ACAAGCACTCCCACTTCATC R: TTCAGCTCGTACTGCATGTC
<i>COL1A1</i> (Collagen type I alpha 1 chain)	F: CGGTGGTTTCTTGGTCGGT R: GTGCGATGACGTGATCTGTGA
<i>BGLAP</i> also known as <i>OCN</i> (Osteocalcin)	F: TGAGAGCCCTCACACTCCTC R: CGCCTGGGTCTCTTCACTAC
<i>VEGFA</i> (vascular endothelial growth factor A)	F: ATCTTCAAGCCATCCTGTGTGC R: GCTCACCGCCTCGGCTTGT

DNA content, ALP activity and collagen quantification

Cell Proliferation Assay Kit CyQUANT® (Thermo Fisher, Landsmeer, The Netherlands) was used following the manufacturer's instructions to measure the total DNA content per scaffold on days 7, and 28 of culture. Samples were washed three times with PBS and stored overnight at -80 °C. Thereafter, samples were freeze-thawed three times. Lysis buffer composed of 0.1 M KH₂PO₄, 0.1 M K₂HPO₄, and 0.1% Triton X-100 (pH 7.8) was added to each sample and incubated for 1 h at room temperature. To assess the ALP activity, 10 µL of the lysed sample was added in triplicate to a white 96-well plate. Next, 40 µL of a CDP-star reagent (Roche Applied Science, Penzberg, Germany) was added to the sample and incubated for 15 minutes in the dark. Absorbance was measured at 466 nm. Obtained values were normalized to the DNA content. The remaining samples were treated with 1mg/ml proteinase K (Merck, Darmstadt, Germany) solution and incubated overnight at 52 °C while shaking. Samples were then freeze-thawed three times and a lysis buffer containing RNase A (Thermo Fisher, Landsmeer, The Netherlands) was added in a ratio of 1:1 to each sample. Finally, 100 µL of the digested sample was added to a black 96 well plate followed by further addition of 100 µL of the GR-dye solution. After 15 minutes of incubation, fluorescence was measured at emission wavelength 520 nm and excitation wavelength 480 nm. To measure the total collagen content in the samples, a Collagen Assay Kit (Merck Darmstadt, Germany) was used. The proteinase K digested samples were used for this purpose, and the obtained results were normalized to the DNA content. The assay reaction mix was prepared by mixing the provided buffer and digest enzyme and added to the samples in a 96-well plate following the manufacturer's instructions. After 60 minutes of incubation at 37 °C, a dye reagent was added and further incubated for 10 minutes at 37 °C. After that, a developer solution was added to all wells and further incubated for 10 minutes at 37 °C. Finally, fluorescence was read at an excitation of 375 nm and an emission of 465 nm.

Tubule formation assay

Human umbilical vein endothelial cells (HUVECs) (Lonza, Basel, Switzerland) were cultured in an endothelial growth medium (EGM, Lonza, Basel, Switzerland) without VEGF supplementation at 37 °C and 5% CO₂. A tubule formation assay was performed in a 96 well µ-Plate (ibidi, Gräfelfing, Germany). Wells were coated with 10 µL of Geltrex™ (Thermo Fisher Landsmeer, The Netherlands) per well, according to the manufacturer's instructions. HUVECs at passage 3 were incubated with Calcein, AM (Thermo Fisher Landsmeer, The Netherlands) for 20 minutes prior seeding at a density of 40 000 cells/cm² in EGM, serving as a control, with EGM + 200 ng/mL VEGF peptide (Milenyi Biotec, Bergisch Gladbach, Germany) serving as positive control, EGM + αMEM collected from the culture of hMSCs on each scaffold for 7 and 28 days (75:25). After 6 hours of incubation,

cells were imaged using an automated inverted Nikon Ti-E microscope (Nikon Instruments Europe BV, Amsterdam, The Netherlands), equipped with a Lumencor Spectra light source (Oregon, USA), an Andor Zyla 5.5 sCMOS camera (Oxford Instruments, Abingdon, United Kingdom), and an MCL NANO Z200-N TI z-stage (Mad City Labs GmbH, Zurich, Switzerland). Tubular parameters were quantified using Fiji software with Angiogenesis analyser Plug-in (U.S. National Institutes of Health, USA).

Detection of specific cytokines using a multiplex assay

Human ProcartaPlex 10-plex (Invitrogen, Amsterdam, The Netherlands) multiplex assay was used to measure concentrations of several anti-, pro-inflammatory as well as pro-angiogenic cytokines and chemokines in the supernatant of direct culture of hMSCs on different 3D composites scaffolds. A list of the analyzed cytokines is presented in **Table 2**. Supernatants were collected on days 7 and 28. At each time point, samples (n=3) were used to measure the average concentrations in duplicates. Samples were diluted 2x in the provided universal assay buffer (based on preliminary experiments). The assay was performed following the manufacturer's instructions. Briefly, capture beads were added to the supplied 96-well plate. The wells were washed twice using a magnet to secure the beads in the wells. Then, samples and antigen standards were added, and the plate was sealed, shaken for 30 mins at room temperature, then transferred to 4°C and stored on a level surface. After an overnight incubation, the plate was shaken for an additional 30 minutes at room temperature. The washing step was repeated twice. Next, a provided mix of antibodies was added into each well and the plate was sealed and incubated for 30 minutes. Then, the plate was washed twice, and Streptavidin-PE was added for another 30 minutes while shaking. The plate was washed twice, and the beads were resuspended in a reading buffer. The data was acquired using the Luminex 200 xPONENT 3.1 instrument (Invitrogen, Amsterdam, The Netherlands). Data was analyzed using the ProcartaPlex Analysis App, a software available through the Connect cloud-based platform of Thermo Fisher Scientific (Landsmeer, The Netherlands). The results are reported as raw data as well as normalized to the DNA content measured with CyQUANT DNA assay.

Table 2. List of analytes measured with ProcartaPlex 10-plex multiplex assay and their abbreviations.

ABBREVIATION	FULL NAME
IDO	Indoleamine 2,3-Dioxygenase
IL-10	Interleukin 10
INF-γ	Interferon-gamma
TNF-α	Tumour necrosis factor-alpha
CCL5	C-C motif chemokine ligand 5
IL-6	Interleukin 6
IL-1β	Interleukin 1 beta
CCL2	C-C motif chemokine ligand 2

MMP-8	Matrix metalloproteinase-8 collagenase
VEGF-A	Vascular endothelial growth factor A

Statistical analysis

All the results are expressed as means $\pm$ standard deviation. The statistical analysis was performed with GraphPad Prism 10 (GraphPad software, California, USA). After assessing the normality of data distribution with the Shapiro-Wilk test, the statistical significance was determined with parametric ANOVA with Tukey's multiple comparison test. Otherwise, non-parametric tests were performed. qPCR results were analyzed with ordinary one-way ANOVA followed by Dunnett's multiple comparison test. Tubule formation assay quantification results, DNA and ALP activity quantification, as well as multiplex data were analyzed with two-way ANOVA followed by Tukey's multiple comparison test. Levels of statistical significance are indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Results and Discussion

Composite preparation and characterization

After ball milling treatment, the particle size was assessed using SEM imaging, showing a final size range of 1–10 μm (**Figure 1A** and **Figure S1A**). The EDX analysis displayed peaks for Si, Ca, and Na (**Figure 1B**), confirming that the glasses mainly consist of SiO_2 , Na_2O , and CaO . The spectrum did not show peaks corresponding to P ions, confirming the absence of detectable amounts of P_2O_5 in the BGs composition.

Subsequently, composites of PCL/BG-WS and PCL/BG-YS were produced according to a blending method [25], incorporating 20% (w/w) of BG, and their final chemical composition was characterized via XRD and FT-IR. The XRD diffraction pattern (**Figure 1C**) revealed the presence in the composites of the two PCL peaks, at 2θ values of 20.3° and 22.6° , corresponding to the (110) and (200) crystallographic planes, respectively [30], and the broad peak of the amorphous silica, at $2\theta = 30.1^\circ$ [20,23]. The FT-IR analysis confirmed both the presence of PCL and BG groups in the composite materials. The stretching vibrations of CH_2 were identified at 2943 cm^{-1} and 2862 cm^{-1} , and the C=O stretching was detected at 1725 cm^{-1} (**Figure S1B**) [30]. Additionally, the BG powders contributed to a broad deflection in the baseline of the transmittance curve around 897 cm^{-1} and 492 cm^{-1} (**Figure 1D**), corresponding to the Si-O-Si symmetric stretching and to the Si-O-Si bending, respectively [31].

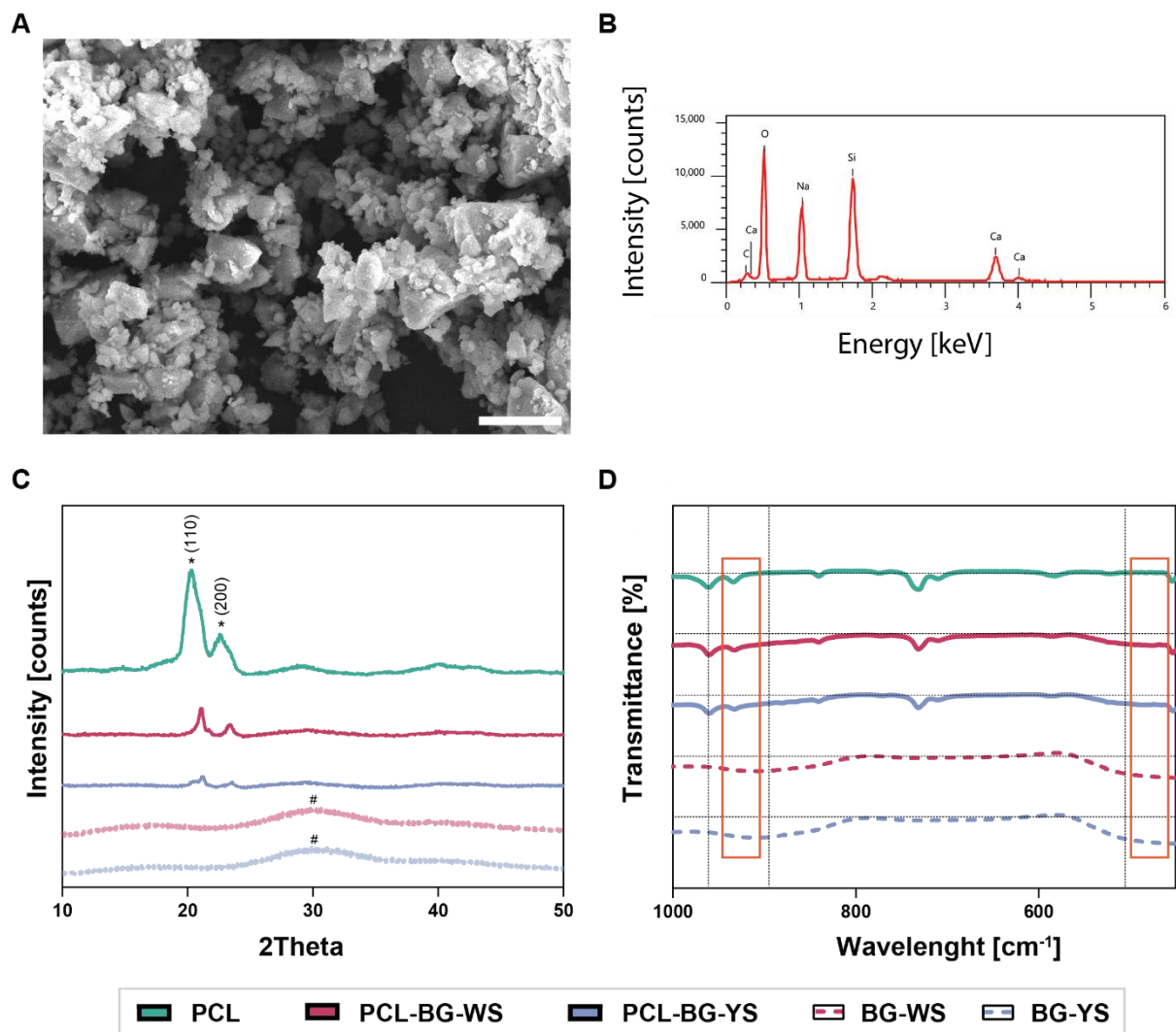


Figure 1. Representative SEM image of BG-WS powder after ball milling. Scale bar = 5 μm (A). EDX analysis of BG-WS powder showing the presence of Si, Na and Ca as main elements (B). XRD of PCL, powders and composites. PCL peaks are visible at 2θ values of 20.3° (110) and 22.6° (200), amorphous silica peak shown at $2\theta = 30.1^\circ$. (C). Zoom of the FTIR spectra between $1000\text{--}400\text{ cm}^{-1}$ of PCL, powders and composites, displaying Si-O-Si stretching (897 cm^{-1}) and bending (492 cm^{-1}) (D).

Scaffold fabrication and characterization

Porous PCL, PCL/BG-WS and PCL-BG/YS scaffolds were successfully fabricated with an in-house developed extrusion-based printer (**Figure S2A**). The constructs were designed to have a star shape pore geometry as shown in the microscopy images and the CAD model view (**Figure 2A-C** and **Figure S2B-D, G**). This particular pore shape was selected based on evidence that the cells commit towards an osteogenic phenotype when cultured on acute-angled pores, more than on the traditional woodpile structure [32]. The morphological features such as fiber diameter, strand-strand distance, and layer thickness displayed values comparable to the theoretical ones of the model, indicating a good printing

fidelity (**Figure S2L**). The BG particles were homogenously distributed in the scaffold fibers (**Figure 2D** and **Figure S2E**) and in their cross-section (**Figure 2E**).

Notably, previous studies reported that inorganic particles were covered by a thin polymer layer when produced with AM, leading to reduced/delayed biological effects [20,33]. Therefore, all scaffolds were subjected to a surface treatment with 5M NaOH. The formation of cracks on the scaffolds' surface was observed, and the BG particles were exposed, confirming the success of the etching treatment (**Figure 2F**, **Figure S2F**).

Subsequently, the mechanical properties of the NaOH-treated scaffolds was evaluated upon compression. A behavior typical for scaffolds was observed, comprising an initial elastic region, followed by a plastic deformation phase. A second increase in the modulus was recorded at a higher strain, as a result of the closure of the pores (**Figure 2G**). Both PCL/BG-WS and PCL/BG-YS scaffolds displayed, in the linear region of the stress-strain curves, higher compressive moduli compared to the PCL only ones, reaching values of 41.66 ± 7.73 MPa and 49.40 ± 2.95 MPa, respectively (**Figure 2H**). This was likely a result of the presence of 20% w/w of bioactive glasses powders in the composites, which reinforced the PCL matrix. Similar behavior was observed in other studies where the addition of 10% w/w or 20% w/w of bioactive glass particles to a PCL matrix induced a significant increase of the compressive moduli [20,34].

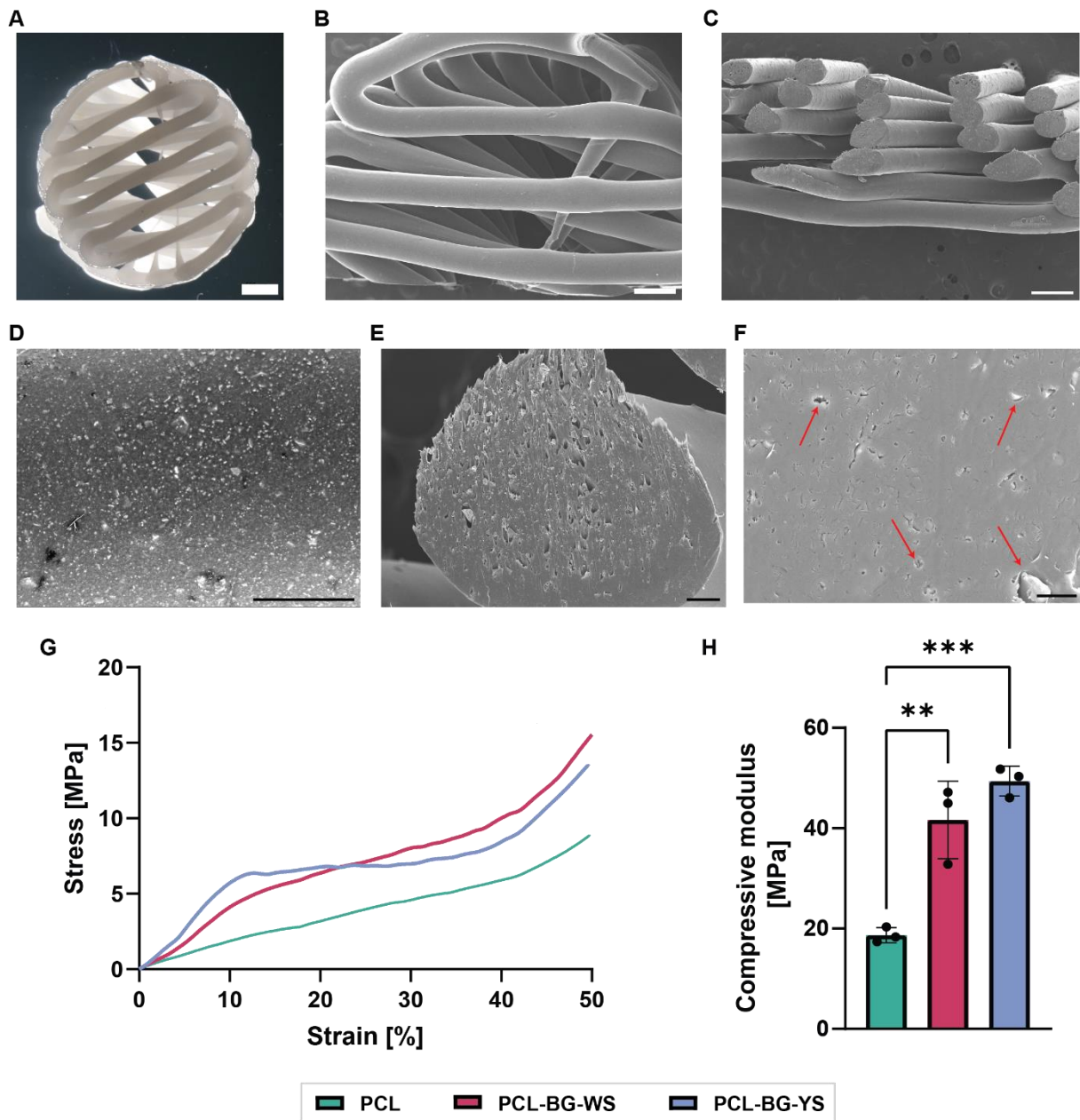


Figure 2. Representative stereomicroscope image of a PCL scaffold with star-shaped pores. Scale bar = 1000 μm (A). Representative SEM image of PCL/BG-WS composite scaffold. Scale bar = 500 μm . Top view (B) and cross section (C). SEM image of PCL/BG-WS fiber surface, displaying homogenous particle distribution. Scale bar = 50 μm (D). SEM image of PCL/BG-WS fiber cross section, showing a uniform particle distribution. Scale bar = 50 μm (E). Zoom on the PCL/BG-WS fiber surface after NaOH treatment, showing the presence of cracks (indicated by the red arrows) on the surface of the fibers. Scale bar = 5 μm (F). Stress-strain curves of PCL, PCL/BG-WS and PCL/BG-YS scaffolds (G). Compressive moduli of PCL, PCL/BG-WS and PCL/BG-YS scaffolds (H). Levels of statistical significance represented by ** $p < 0.01$, *** $p < 0.001$.

Scaffold degradation and ion release

After scaffold fabrication and characterization, the degradation of PCL, PCL/BG-WS, and PCL/BG-YS samples after NaOH treatment was investigated in a physiological-like

environment, by supplementing PBS with lipase enzyme. This enzyme is able to degrade PCL scaffolds [28]. Therefore, the weight loss percentage of the scaffolds over time was calculated, up to 28 days of incubation (**Figure 3A**). All the scaffolds showed an approximately constant weight when incubated in only PBS. However, in PBS supplemented with the enzyme, PCL/BG-YS scaffolds displayed a significant weight loss after 7 days and PCL/BG-WS after 14 days. At 28 days all the samples reached a total weight loss percentage of $5.61 \pm 0.41 \%$, $4.64 \pm 0.45 \%$, and $5.54 \pm 1.52 \%$ for PCL, PCL/BG-WS and PCL/BG-YS, respectively. The presence of the enzyme contributed to the accelerated degradation, as previously reported in the literature. For instance, Martins et al. showed degradation of PCL scaffolds around 5-7% after 30 days of immersion in PBS containing 110 U/L of lipase [28]. Duarte et al. incubated PCL scaffolds in PBS with lipase 102 U/l for 21 days while shaking, recording a weight loss percentage between 10-20% [35].

In parallel to the scaffold degradation, the cumulative release of two biologically relevant ions, Si and Ca, from PCL/BG-WS and PCL/BG-YS scaffolds was recorded over 28 days of degradation. The release of Si and Ca ions from bioactive glasses plays a crucial role in osteogenesis and angiogenesis. A literature review reported several studies showing that the synergistic effect of these ions induced an increase in metabolic activity, ALP activity and VEGF production [36]. In both PBS only and PBS containing lipase, an increasing concentration of Si and Ca ions was observed, with higher values in the presence of the enzyme. The release of Si reached the highest concentration of 117.0 ± 20.43 ppm, for PCL/BG-YS scaffolds in PBS with lipase, with a burst release of 50.66 ± 20.43 ppm in the first 24h (**Figure S3A**). The amount of Ca ions reached a much lower cumulative release of 9.13 ± 0.07 ppm, and no initial burst release (**Figure S3A**). The higher ion release observed for the PCL/BG-YS composites in the presence of lipase may be attributed to the inhomogeneous and variable nature of the enzymatic degradation of the PCL matrix, which might have resulted in a different exposure of the embedded BG particles between the two composites.

In addition, trace levels of Ti and Fe ions were detected (**Figure S4**). The cumulative release of these elements did not exceed 0.1 ppm for Ti ions and 0.14 ppm in the case of Fe ions. This low release was expected, as both elements are present in the BGs only at trace concentrations. Nevertheless, the release of these ions could still be detected, indicating measurable ion exchange between the composites and the surrounding fluid. Notably, these ions are known to exert biological effects even at very low concentrations [37,38].

Typically, the release of ions from the BGs leads to an increase in pH of the culture medium. Already a slightly basic pH of the medium has been shown to result in cell death during cell culture [20,39]. Therefore, prior to cell seeding, PCL, PCL/BG-WS, and PCL/BG-YS were incubated in cell culture medium for 3 days, measuring the pH every day. In the

first 48h of incubation, a significant increase in the pH values in the media incubated with composite scaffolds was measured (**Figure 3C**), implying even higher local pH values directly at the scaffold interface where the ions are released from [39]. The pH variation was also qualitatively confirmed by a change of color of the medium, which turned bright pink after 24h (**Figure S3B**). On the third day, a stabilization of the pH values around 7.8 was observed. The results suggested that, prior to cell seeding, the scaffolds should be incubated in media for 3 days to maintain a physiological pH during cell culture and prevent cell death. A similar strategy was also followed by other groups, who performed pre-incubation of 3 to 7 days prior cell seeding, to bring pH values from above 8 in the first hours to physiological levels [40–42].

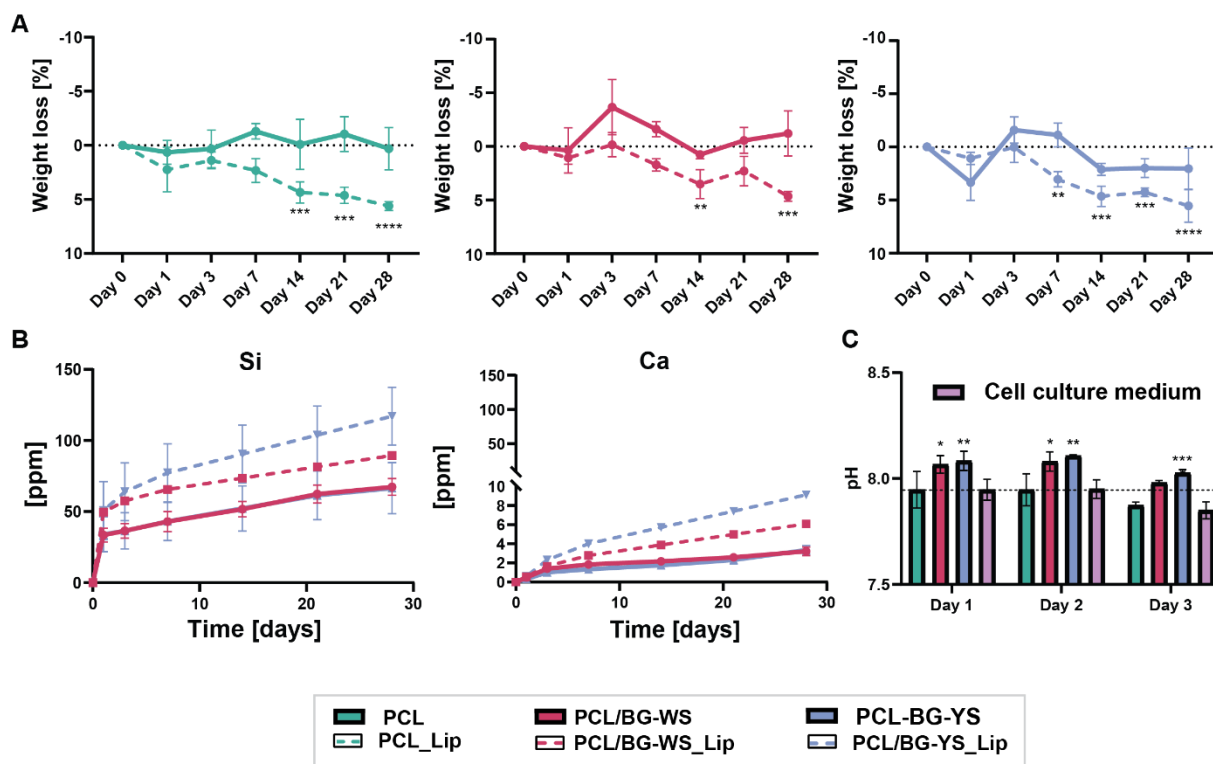


Figure 3. Degradation in PBS or PBS + Lipase of PCL, PCL/BG-WS and PCL/BG-YS scaffolds for 28 days (A). Cumulative release of silica and calcium ions from PCL/BG-WS and PCL/BG-YS scaffolds in PBS and PBS + Lipase for 28 days (B). pH variation of basic culture medium incubated with PCL, PCL/BG-WS and PCL/BG-YS scaffolds over 3 days, without enzyme (C). Values were compared to the cell culture medium. Levels of statistical significance represented by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Angiogenic properties

Angiogenic properties of the scaffolds were assessed by analyzing the *VEGFA* gene expression (**Figure 4A**) of hMSCs cultured directly on scaffolds for 7 and 28 days, quantification of the VEGFA secreted into the cell culture medium (**Figure 4B**), as well as

through an assessment of tubule-like structure formation of HUVECs cultured in medium conditioned by the scaffolds for 7 or 28 days (**Figure 4C**).

While at day 7 the gene expression of *VEGFA* was comparable among conditions, at day 28 its expression was upregulated on PCL/BG-WS composites compared to PCL scaffolds (fold change value of 1.43 ± 0.17). Interestingly, on the protein level, VEGFA concentrations were significantly higher at day 7 than at day 28 on all investigated scaffolds (**Figure 4B**), which suggests that the upregulation of the gene expression might have occurred at an earlier time point than investigated in this study. Furthermore, at both time points the concentrations of VEGFA were significantly higher on PCL/BG-WS and PCL/BG-YS scaffolds when compared to scaffolds comprising PCL alone. Additionally, tubule-like structure formation by HUVECs exposed to a conditioned medium containing dissolution products of each investigated scaffold and the corresponding quantification of the tubular parameters are shown in **Figure 4C**. HUVECs, as well as other endothelial cells, will grow on a soft substrate and form tubular networks ranging from short and isolated segments to highly developed meshing, depending on the treatment conditions [43]. In other words, the more developed the mesh, the more advanced the angiogenesis. Therefore, this is a very commonly used *in vitro* angiogenesis assay used to identify inhibitors or stimulators of angiogenesis [44]. In this study, tubular structures formed in each condition with a slightly higher number of junctions and total length quantified in the case of cells exposed to medium collected at day 7 than at day 28 for PCL/BG-WS. Mean mesh size, however, was significantly higher for cells exposed to medium collected at day 28 compared to day 7 for PCL/BG-YS scaffolds. Additionally, at day 28 both composite scaffolds (PCL/BG-WS and PCL/BG-YS) exhibited significantly greater mean mesh sizes ($8136.5 \pm 2376.9 \mu\text{m}^2$ and $8103.0 \pm 1548.6 \mu\text{m}^2$, respectively) compared to PCL alone. No statistically significant differences were observed compared to the EGM and EGM+VEGF controls (**Figure S5**).

The observed enhanced angiogenic effect in both composite scaffolds may be attributed to the presence of BGs, as it has been previously reported that silicate-based glasses have been linked with induction of angiogenic differentiation and upregulation of genes related to angiogenesis [14,16]. BG dissolution products, in particular Si ions, have been shown to promote angiogenic signaling and endothelial cell organization [45]. Additionally, the distinct trace element profiles of the two BGs, higher Ti ions content in BG-WS and higher levels of Fe and Al in BG-YS might have contributed synergistically to the observed angiogenic effects [14,19,46]. However, the cumulative release of Ti and Fe from both PCL/BG-WS and PCL/BG-YS composite scaffolds was comparable and remained within the trace element range. Low concentrations were anticipated, since these elements are present in the BGs only as minor elements and the release measurements were performed on composites containing 20 wt% BG within the PCL matrix.

Previous studies investigating angiogenic properties of PCL/BG composites including those based on SiO₂-Na₂O-CaO BGs [20] or Boron-containing BGs (B-BGs) [10] reported earlier *VEGFA* gene upregulation by adipose-derived hMSCs and rat MSCs, respectively. Regarding the results at the protein level, Gerhardt and colleagues [47] observed an increased VEGFA secretion by fibroblasts on 45S5-BGs in PDLLA composite films compared to polymer-only films, which is in line with our observations of higher VEGFA concentrations in composite scaffolds. Other research groups have also reported positive effects on tubule formation by HUVECs cultured with dissolution medium from PCL-based composites. For example, a study using a 3D PCL scaffold containing a hypoxia-inducing factor demonstrated accelerated vascular pattern formation compared to PCL-only control scaffolds [7]. Although this study reported significantly higher total length and number of junctions, the counts were much lower than those observed in our study. Specifically, the highest number of junctions reported was approximately 40, whereas we observed up to 122.8 ± 37.4 junctions. Similarly, the total length in their study was approximately 3000 μm , while in our study it was $10,111.8 \pm 1866.4$ μm . These reported differences might suggest that the experimental conditions or scaffold composition in our study may have been more favorable to vascularization, potentially reflecting the effectiveness of our approach in promoting more robust tubule formation.

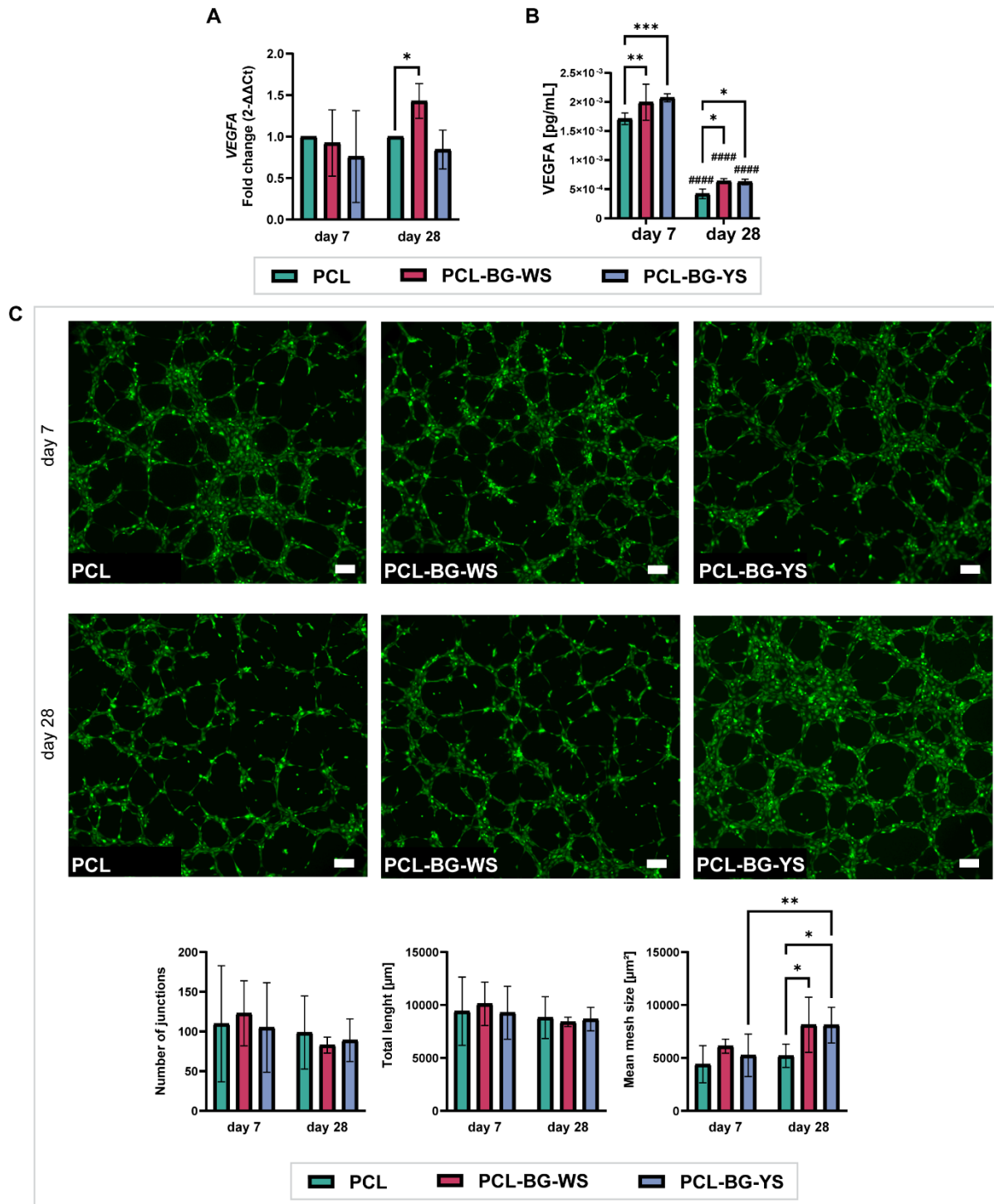


Figure 4. Angiogenic properties of 3D scaffolds. (A) Expression of *VEGFA* by hMSCs cultured directly on 3D scaffolds for 7 and 28 days. (B) The concentration of *VEGFA* measured in the culture medium after 7 and 28 days of culture. (C) Tubule-like structure formation of HUVECs cultured with medium collected from a direct culture of hMSCs on 3D scaffolds for 7 days or 28 days and quantification of selected tubular parameters: number of junctions, total length, and mean mesh size. Scale bar = 200 μm. Green, fluorescent staining corresponds to Calcein-AM. Levels of statistical significance are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

Osteogenic properties

To evaluate the osteogenic properties of the developed PCL/BG scaffolds, the expression of osteogenic gene markers (**Figure 5**, **Figure S6**), ALP activity (**Figure 6A**), collagen production (**Figure 6B**), and ECM formation (**Figure 6C** and **6D**) were assessed in hMSCs cultured directly on the investigated scaffolds after 7 and 28 days.

At day 7 in basic medium, *RUNX2* showed the highest expression, with significant upregulation on PCL/BG-WS and PCL/BG-YS (fold change values of 4.55 ± 0.70 and 5.19 ± 0.56 , respectively) than on PCL scaffolds (**Figure 5**). Culture in the osteogenic medium resulted in a similar expression pattern and values. (**Figure S6**). The approximately 5-fold upregulation of *RUNX2* in both basic and osteogenic media suggests a strong intrinsic effect of both BGs on early osteogenic differentiation. In contrast, Font Telado et al. reported that PCL scaffolds comprising 10% w/w 52S-BG (a BG from the same glass system as those explored in this study) did not induce significant *RUNX2* upregulation [20]. Similarly, Distler et al. [48] found no notable influence on *RUNX2* expression in PLA scaffolds incorporating 1% w/w 45S5-BG. These previous findings indicate that the BG concentrations reported in these composites may have been insufficient to stimulate osteogenic differentiation process. Furthermore, Söhling et al. [49] and Wang et al. [9] incorporated 20%w/w of S53P4-BG, the same amount as used in this study, into PLA and PCL scaffolds, respectively, yet neither reported significant *RUNX2* upregulation. The surface treatment performed on our scaffolds, absent in the studies of Söhling et al. and Wang et al., enabled BG exposure and subsequent ion release, potentially enhancing the observed osteogenic response.

Interestingly, *ALPL* expression was the highest on day 28 in the case of PCL/BG-WS, with a fold change of 4.25 ± 0.37 (**Figure 5**). In contrast, in osteogenic medium, *ALPL* expression was downregulated on composites in comparison with PCL alone at both evaluated time points (**Figure S6**). This notably higher *ALPL* expression observed for PCL/BG-WS may be partially explained by the higher Ti content in BG-WS. Ti ions have previously been reported to positively influence osteogenic differentiation and specifically enhance *ALPL* gene expression [50,51]. Nevertheless, as discussed earlier, the measured cumulative release of Ti ions was comparable between the composites and remained within the trace element range. The low release of Ti from the scaffolds was expected, as it is present in the BG only in trace amounts and the quantifications were performed on composite scaffolds containing 20wt% BGs dispersed within the polymer matrix. Although the cumulative Ti release from both composites was minimal and comparable, Ti may still exert a local effect at the material interface, as previously reported to increase viability and facilitate greater attachment of MC3T3 osteoblasts [37].

At the protein level (**Figure 6A**), ALP activity was overall significantly higher on PCL scaffolds compared to both composites, and the same pattern was observed under

osteogenic conditions (**Figure S8**). Typically, literature studies in the field of ceramic materials report an earlier upregulation of *ALPL* expression, followed by its decrease overtime. This pattern is usually observed when the ions are coated on the polymeric scaffold surface rather than incorporated in the bulk fibers [9]. The majority of these non-covalently coated particles are likely released in the first days of culture, immediately inducing an osteogenic effect. In this study, however, the delayed *ALPL* expression alongside the generally higher ALP activity detected on PCL scaffolds, may be explained by the notably low phosphorous content in the BGs formulation. It is well-established that a high inorganic phosphate concentration significantly induces ALP activation [52,53]. Consequently, the required threshold concentration for activating *ALPL* expression may have been reached only at later time points. Thus, the limited potency of the specific BG formulation in driving *ALPL* expression at both gene and protein levels can likely be attributed to its inherently low phosphorus content.

Regarding the expression of late osteogenic markers, *COL1A1* and *OCN*, which both contribute to matrix formation, several differences were observed (**Figure 5**). Expression of *COL1A1* on day 7 was comparable among all conditions. On day 28, however, it was significantly higher on PCL/BG-WS (fold change 2.17 ± 0.27) than in the case of the other two scaffolds. *OCN* increased significantly between day 7 and day 28 on both PCL/BG-WS and PCL/BG-YS. On day 28, the fold gene expression on PCL/BG-WS reached 3.52 ± 0.10 . Culture with osteogenic medium (**Figure S6**) resulted in a similar expression pattern of *COL1A1*, while the expression of *OCN* was similar on day 7 but downregulated on day 28 in comparison to PCL.

These results reinforce that both the amount of BGs and the release rate in composite scaffolds influences osteogenic outcomes. A significant increase of *OCN* expression after 21 days in basic medium was reported in a study where 50% of 45S5 BG was used [42], while studies with lower glass concentration or slow release did not demonstrate a stimulatory effect on *OCN* and *COL1A1* expression [9,20,42,48,49]. Finally, ECM formation was investigated and reported in **Figure 6C-D**. SEM images of scaffolds cultured with hMSCs for 7 or 28 days revealed matrix production on the top as well as in the cross-section of all three formulations of the scaffolds at both investigated time points. It could be observed that the amount of matrix increased between days 7 and 28 on all scaffold compositions and no particular morphological differences were observed between PCL and PCL/BG-WS, or PCL/BG-YS. Similar observations were made when hMSCs were cultured on scaffolds in osteogenic medium (**Figure S9**). On a protein level, the total production of collagen was the highest at day 7 in the case of culture on PCL scaffolds, while no differences were observed at day 28 (**Figure 6B**). These results suggest that all scaffolds supported sufficient matrix deposition. Based on the genetic evaluation (such as *COL1A1* and *ALPL* being upregulated in PCL/BG-WS at day 28) differences in the biochemical composition of the matrix were

anticipated, yet longer culture periods and more extensive biological evaluations may be required in future studies to confirm the findings.

The differences in osteogenic outcomes observed between PCL/BG-WS and PCL/BG-YS composites may be related, as previously anticipated, to the compositional difference on the trace level elements between the BG-WS and BG-YS. While both BGs were previously synthesized from natural raw materials within the same glass system and at identical PCL:BG ratios, previous analysis revealed that BG-WS contained a higher amount of Ti ions, whereas BG-YS was richer in Al and Fe [23]. Ti is known to enhance osteogenic differentiation by promoting *ALPL* gene expression and ECM mineralization, which may explain the upregulation of *ALPL* and *COL1A1* observed in the PCL/BG-WS group [50,51]. This suggests that even subtle compositional differences in trace elements may significantly affect osteogenic behavior.

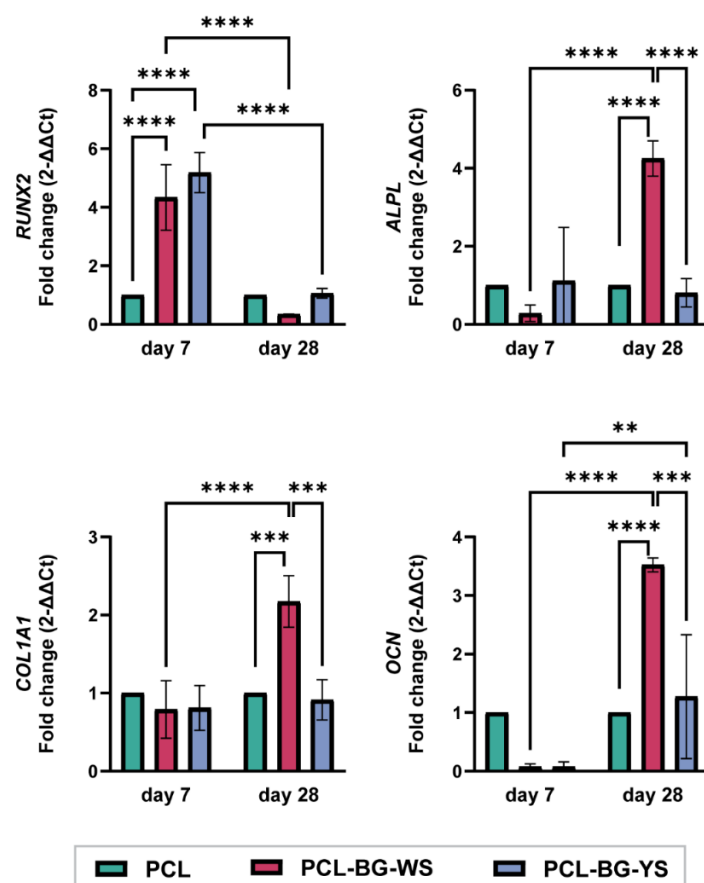


Figure 5. Expression of *RUNX2*, *ALPL*, *COL1A1*, and *OCN* by hMSCs cultured on PCL, PCL-BG-WS, and PCL-BG-YS scaffolds for 7 and 28 days in basic culture medium. Results are shown as fold gene expression, normalized to the expression on the PCL sample using the $\Delta\Delta C_T$ method. Levels of statistical significance are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

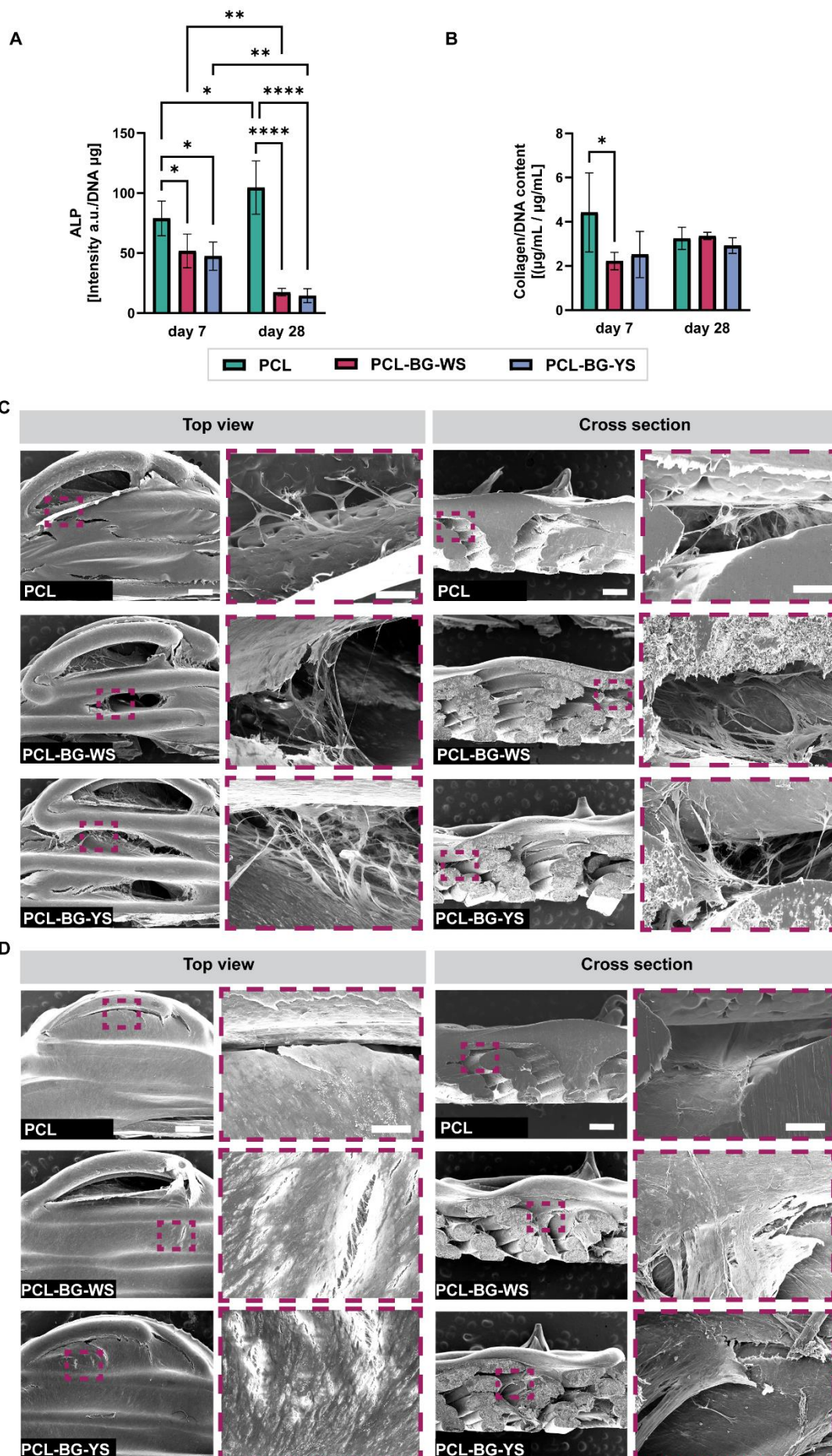


Figure 6. Osteogenic properties of the scaffolds. (A) ALP activity, (B) collagen production, ECM formation on (C) day 7 and (D) day 28 in basic culture medium. Scale bars = 500 μm and 100 μm for the magnified pictures. Levels of statistical significance are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

Influence of the scaffold composition on the secretion profile of hMSCs

To study the effect of the added inorganic particles to composite scaffolds on the paracrine function of hMSCs, several pro- and anti-inflammatory cytokines, chemokines, and proteins (**Table 2**) were measured. The cell culture supernatant of hMSCs cultured on different 3D composites was analyzed using a 10-plex multiplex platform. **Figure 7A** shows a heatmap demonstrating the concentrations of measured analytes on days 7 and 28 normalized to the DNA content, while **Figure 7B** shows a detailed comparison, including statistical analysis, of the analytes that were expressed the most consistently, including CCL2, TNF- α , IL-10. Furthermore, **Figure S10** shows the cytokine concentrations reflecting a more bioengineering perspective of the cytokine release analysis.

Overall, 7 out of 10 measured analytes were detected in all supernatants. Measured concentrations of IL-1 β , MMP-8, and CCL-5 were lower than the lowest concentration on the standard curves for the majority of the samples, and thus outside of the detection range. Of note, the lowest detectable concentration for IL-1 β was 1.6 pg/mL, for MMP-8 it equaled 20 pg/mL, and for CCL-5 was 0,6 pg/mL.

The highest concentrations of the measured analytes were detected in supernatants collected after 7 days of hMSCs cultured on PCL scaffolds. Here, the concentrations of CCL2, TNF- α and IL-10 were significantly higher on day 7 than on day 28 (**Figure 7B**). In contrast, for the composite scaffolds, the concentrations increased between day 7 and 28 or remained constant. For instance, CCL2 was significantly higher after 28 days for both PCL/BG-WS and PCL/BG-YS composites, while IL-10 remained constant. The cytokine concentrations prior DNA normalization (**Figure S10**) followed the same general trend.

These results suggest that on day 7 PCL scaffolds might have triggered inflammatory responses of hMSCs more than the PCL/BG composite scaffolds, while the addition of both BG-WS and BG-YS contributed to reducing the secretion of inflammatory factors. Moreover, the composite scaffolds enhanced the production of pro-angiogenic factors such as CCL2 and the previously mentioned VEGFA. Similar observations were previously reported by Söhling et al. [49], although they employed a different experimental setup using a whole-blood stimulation assay.

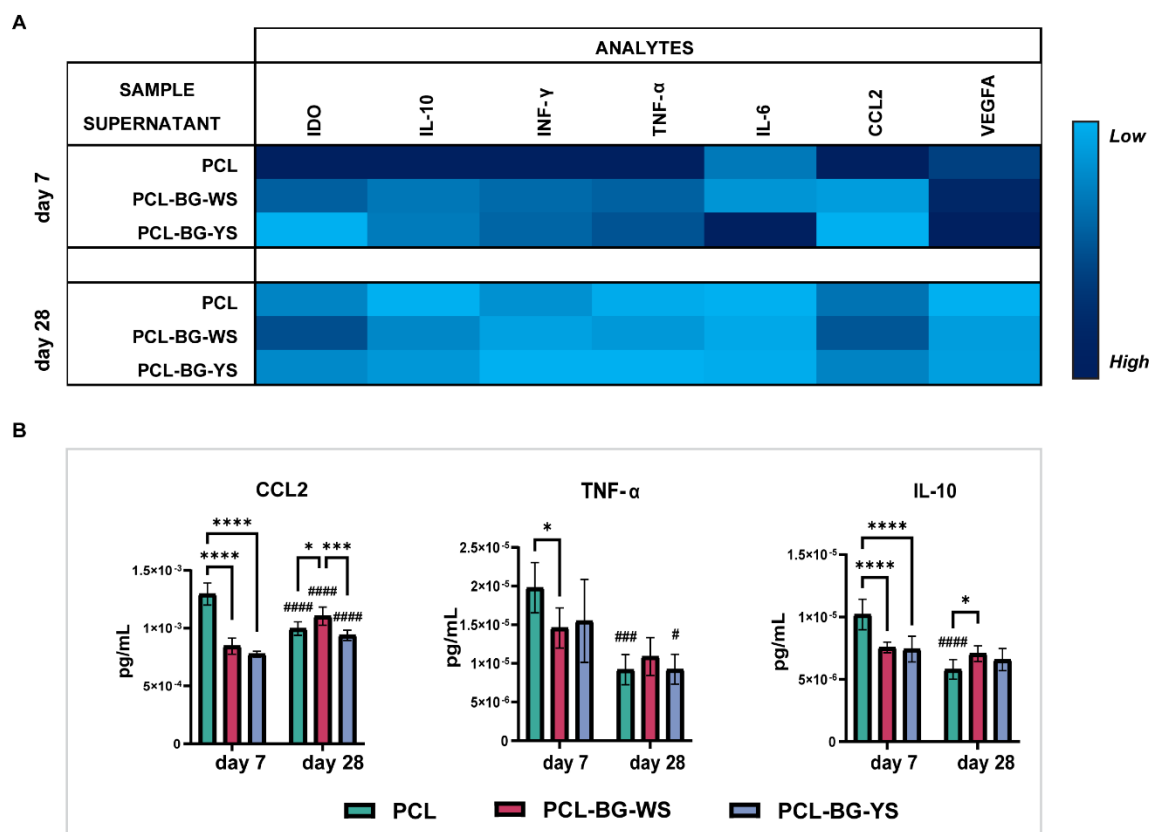


Figure 7. Influence of the scaffold composition on the paracrine function of hMSCs. (A) Heat map demonstrating overall differences in the secretion profiles of hMSCs cultured on different scaffolds (PCL, PCL-BG-WS, and PCL-BG-YS) measured on days 7 and 28. (B) Detailed comparison of measured concentrations of CCL2, TNF- α , and IL-10. Symbol # demonstrates the statistical significance of the comparison between days 7 and 28 of the same type of scaffold, while * indicates statistically significant differences between different samples at the same time point. Levels of statistical significance are indicated by * or # $p < 0.05$, ** or ## $p < 0.01$, *** or ### $p < 0.001$ and **** or #### $p < 0.0001$.

Overall, the results of this study demonstrated that both PCL/BG-WS and PCL/BG-YS possess osteogenic, angiogenic, and potential immunomodulatory properties. The composition of the BGs certainly played a crucial role in influencing the biological performance of the composite scaffolds. PCL/BG-WS exhibited the strongest osteogenic responses among the tested formulations, which may be related to its higher content of Ti ions compared to BG-YS.

In this study, both composites contained P₂O₅-free BG powders based on the SiO₂-CaO-Na₂O system, which were already reported to have osteogenic and angiogenic properties when directly incubated with hMSCs [19]. Here, we demonstrated that also the incorporation of P₂O₅-free bioactive glasses into a PCL matrix led to enhanced osteogenesis and angiogenesis while potentially modulating inflammatory responses. This highlights the potential of these composite materials for bone tissue engineering.

While this study focused on naturally derived P₂O₅-free BG combined with a polymer, it is important to interpret these findings within the broader context of conventional P₂O₅-

containing systems. Most commercially available BGs, such as those based on the 45S5 and S53P4 compositions, contain P_2O_5 . Numerous studies have demonstrated that P_2O_5 -containing BGs exhibit high bioactivity but may degrade rapidly, which may limit the long-term stability and sustained biological activity of composite scaffolds [16–18]. Recent research has also introduced novel P_2O_5 -free BG formulations designed to improve chemical stability and increase the control of ions release, expanding the landscape of BGs for bone regeneration [20,23]. In this context, the naturally derived P_2O_5 -free formulations investigated here (BG-WS and BG-YS) maintained their biological effectiveness when incorporated into PCL while providing a more controlled dissolution behavior. The P_2O_5 -free BGs can complement or provide sustainable alternatives to the existing BGs and BG-based composites for bone regeneration applications.

In conclusion, further investigations should be conducted that include direct co-cultures of hMSCs (or osteoprogenitor cells) and HUVECs on the PCL/BG-WS scaffolds. This would be valuable to understand how cell-to-cell interactions influence osteogenic and angiogenic responses with respect to the composite scaffolds, enabling further refinement of scaffold designs for improved bone regeneration, including vascularization. An assessment of these scaffolds *in vivo*, in injury models where a good vascularization is of utmost importance, should be considered as an ultimate step towards enlightening the translational potential of this composite-based scaffold.

Conclusions

This study demonstrates that the presence of bioactive glasses in composite materials enhances osteogenic and angiogenic properties of the scaffolds. This was reflected by the upregulated expression of early and late osteogenic markers as well as enhanced matrix deposition. Additionally, the composites stimulated the secretion of pro-angiogenic factors, while limiting inflammatory processes, in comparison with PCL only scaffolds. The developed composite material, here successfully fabricated into a 3D scaffold, represents a promising advancement in bone substitute materials, offering new potential for enhanced bone regeneration and improved outcomes in tissue engineering. Future investigations should focus on long-term *in vivo* validation and optimization of BG composition to further enhance regenerative outcomes.

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CHAPTER 4

Supplementary information

Additive manufacturing and in vitro characterization of scaffolds consisting of PCL/P₂O₅-free bioactive glasses composites with angiogenic and osteogenic potential

Martyna Nikody, Sophia Dalfino*, Pamela Habibovic, Lizette Morejón, José A. Delgado, Gianluca Martino Tartaglia, Claudia Dolci, Elizabeth R. Balmayor, Lorenzo Moroni*

Submitted and under revision at Biomaterials (Elsevier)

**These authors contributed equally to this manuscript*

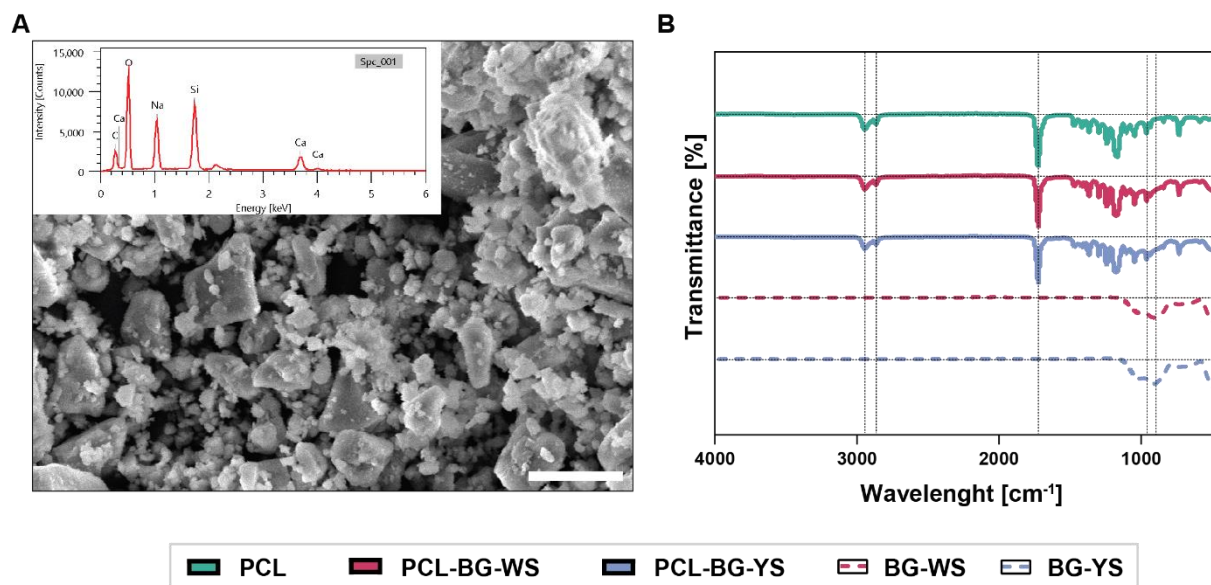


Figure S1. Representative SEM image and EDX analysis of BG-YS powders after ball milling. Scale bar = 5 μm (A). Full FTIR spectra of PCL between 4000-400 cm^{-1} , powders and composites, displaying CH_2 (2943 cm^{-1} and 2862 cm^{-1}) and the C=O (1725 cm^{-1}) stretching (B).

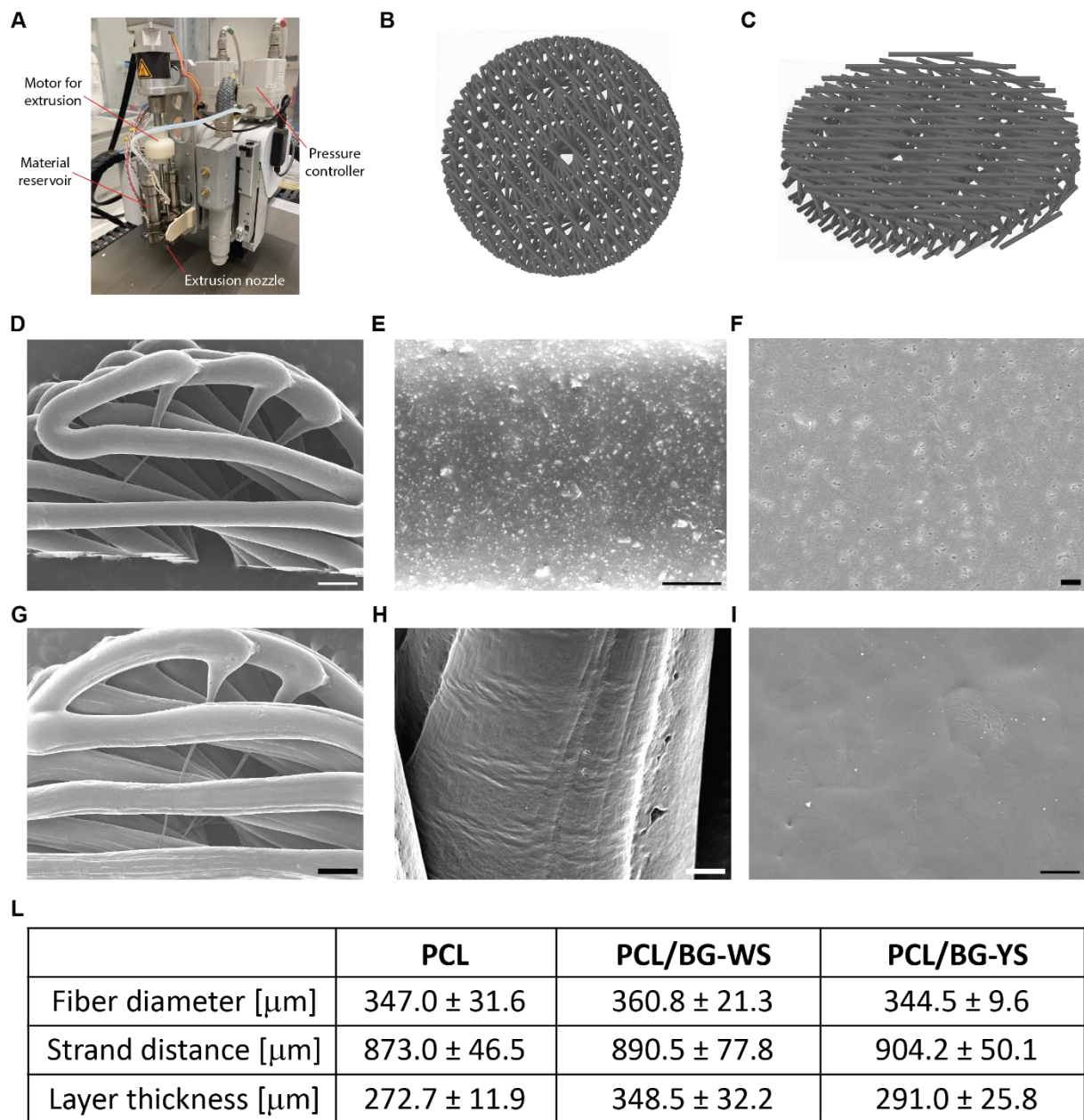


Figure S2. In-house developed printing setup (A). CAD model of the star-shape scaffold, showing a top (B) and a lateral (B) view. Representative SEM image of PCL/BG-YS composite scaffold. Scale bar = 500 μm (D). SEM image of PCL/BG-YS fiber surface, displaying homogenous particle distribution. Scale bar = 50 μm (E). Zoom on the PCL/BG-YS fiber surface after NaOH treatment, showing the presence of cracks on the surface of the fibers. Scale bar = 5 μm (F). Representative SEM image of PCL composite scaffold. Scale bar = 500 μm (G). SEM image of PCL fiber surface. Scale bar = 50 μm (H). Zoom on the PCL fiber surface after NaOH treatment, showing the presence of cracks on the surface of the fibers. Scale bar = 5 μm (I). Morphological parameters of the scaffolds: fiber diameter, strand distance and layer thickness (L).

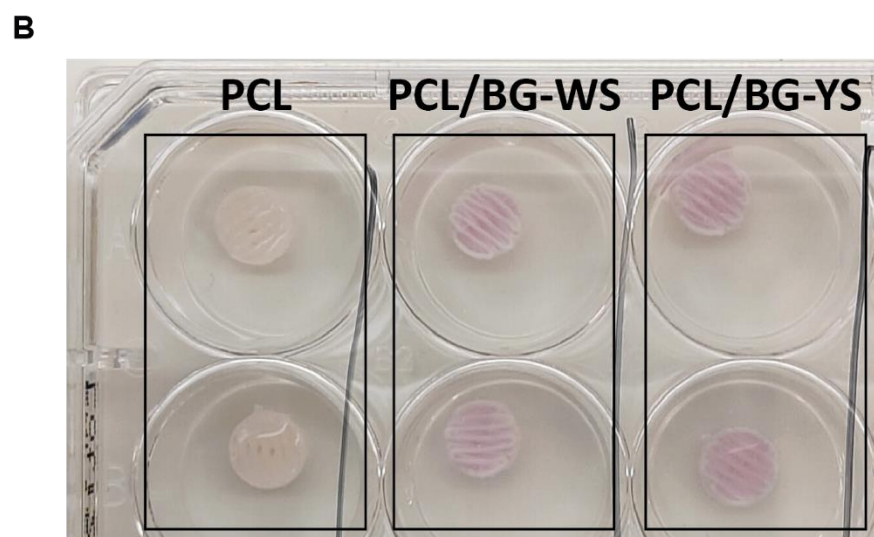
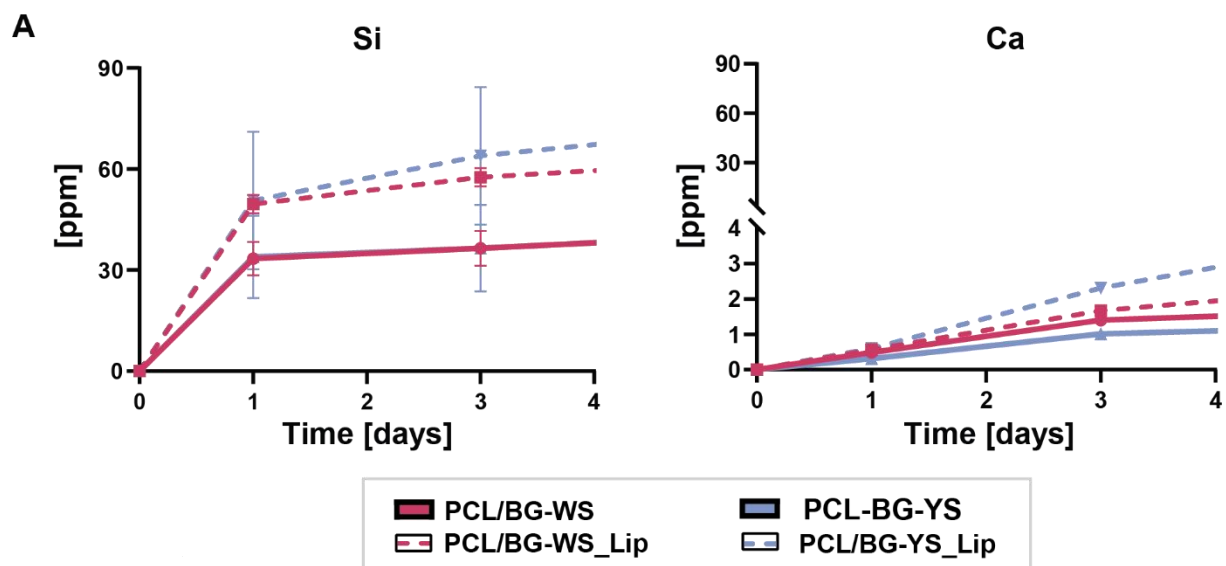


Figure S3. Ion release in the first days of incubation in medium showing burst release for Si and linear release for Ca (A). Pictures of the scaffolds after 1 day in cell culture medium, showing more pink color for the composite scaffolds where the pH increased (B).

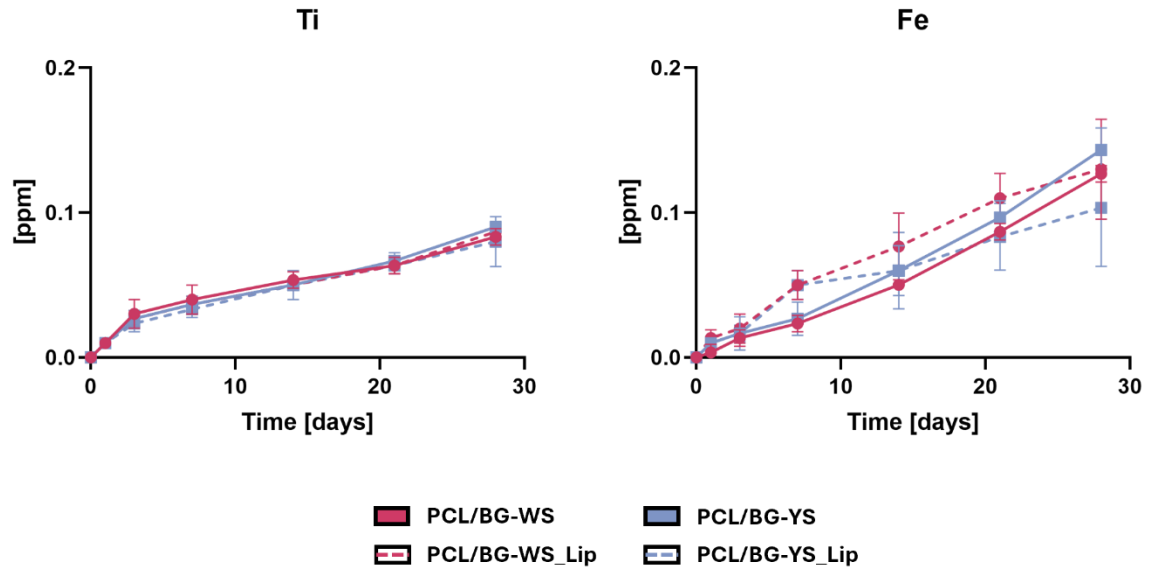


Figure S4. Cumulative release of titanium and iron ions from PCL/BG-WS and PCL/BG-YS scaffolds in PBS and PBS + Lipase for 28 days.

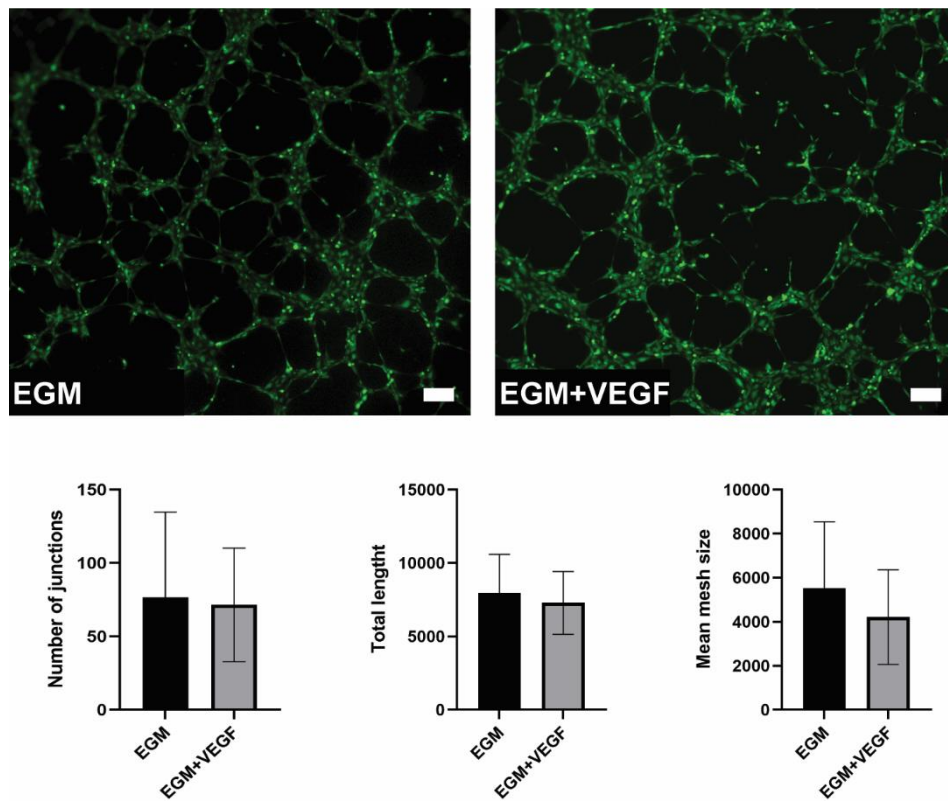


Figure S5. Controls for the tubule formation assay. Representative images and quantification of tubular parameters of HUVECs cultured with EGM medium alone or EGM supplemented with VEGF. Scale bar = 200 μ m. Green fluorescence staining corresponds to Calcein-AM.

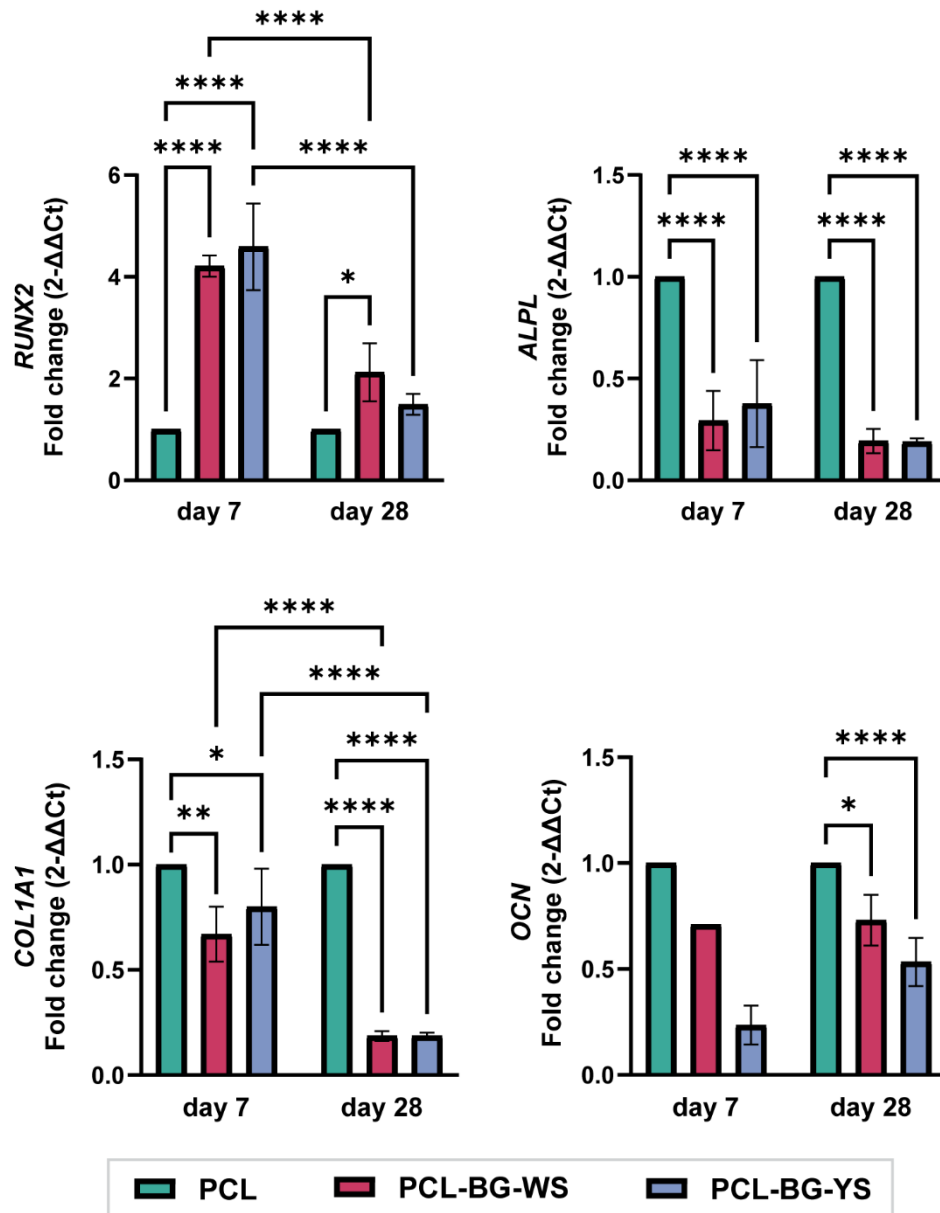


Figure S6. Expression of *RUNX2*, *ALPL*, *COL1A1*, and *OCN* by hMSCs cultured on PCL, PCL/BG-WS, and PCL/BG-YS scaffolds for 7 and 28 days in osteogenic culture medium. Results are shown as fold gene expression, normalized to the expression on the PCL sample using the $\Delta\Delta C_t$ method. Levels of statistical significance are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

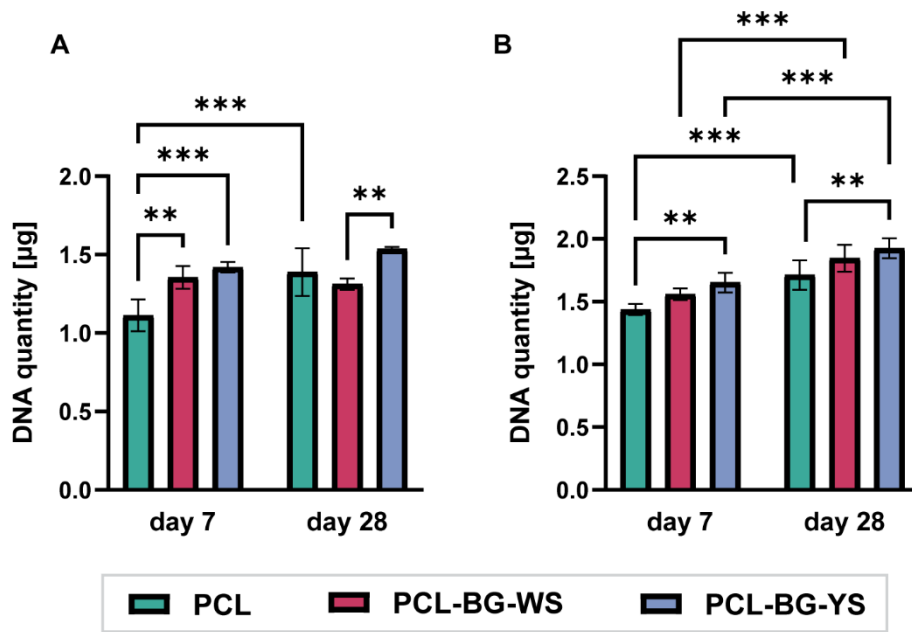


Figure S7. Proliferation of hMSCs quantified on days 7 and 28 when cultured on 3D scaffolds in (A) basic and (B) osteogenic medium. Levels of statistical significance are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

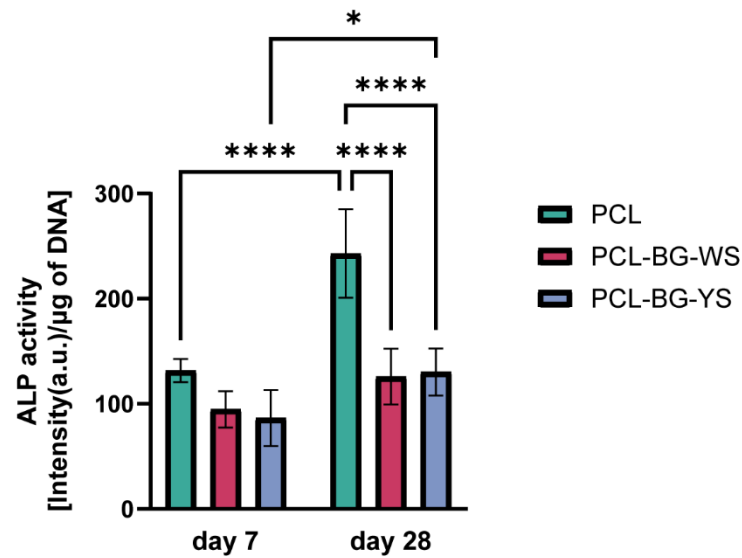


Figure S8. ALP activity in osteogenic medium normalized to the DNA content. Levels of statistical significance are indicated by * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

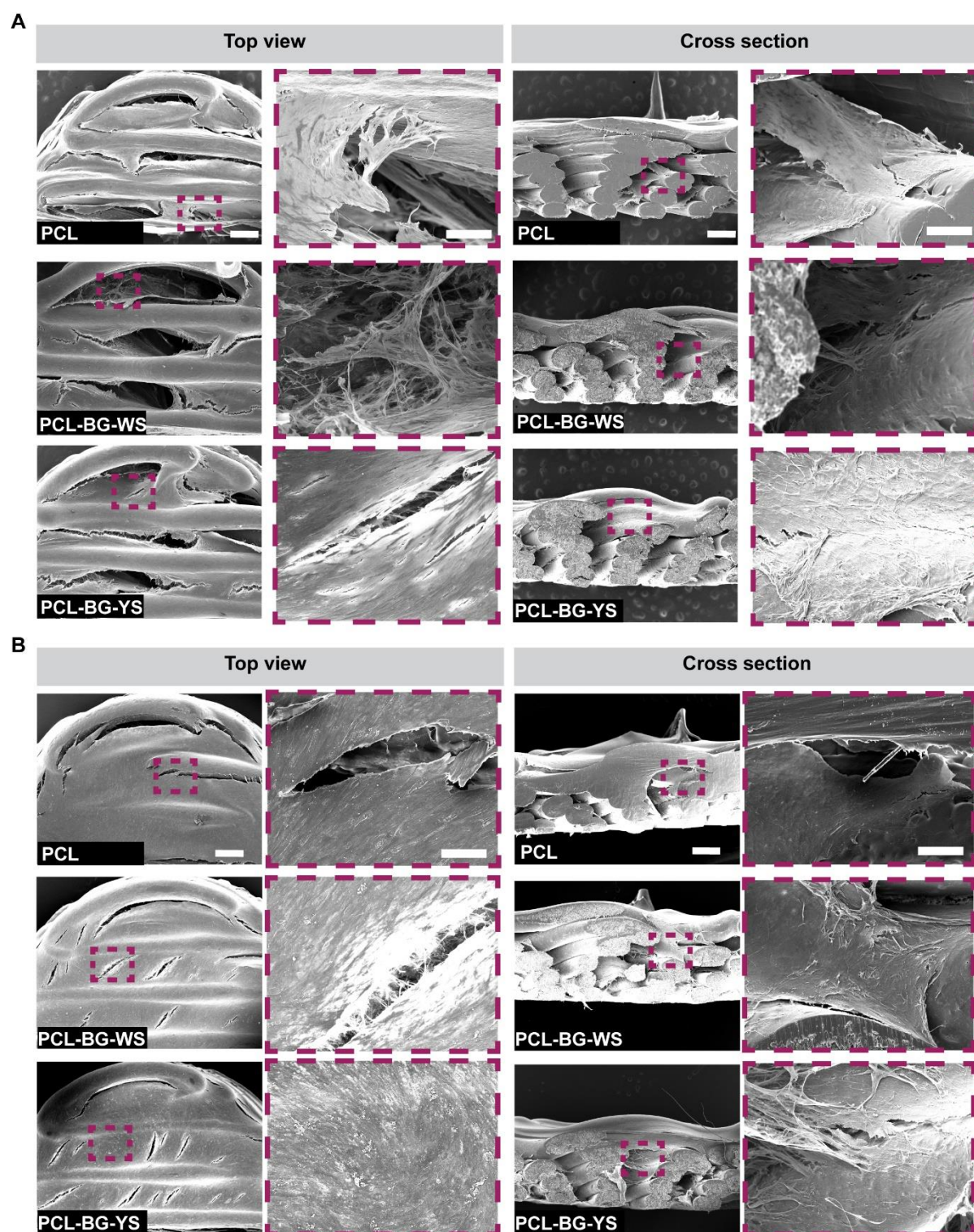


Figure S9. ECM formation on (A) day 7 and (B) day 28 in osteogenic medium. Scale bars = 500 μ m and 100 μ m for the magnified pictures.

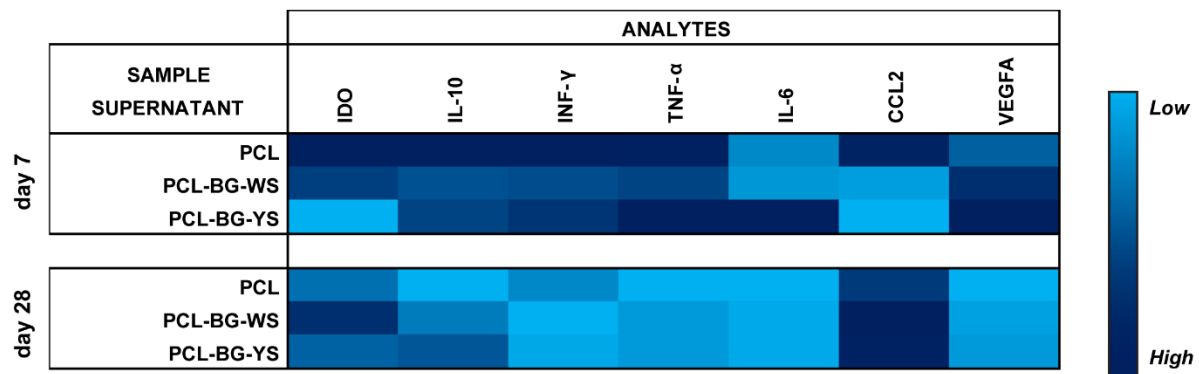


Figure S10. Influence of the scaffolds' composition on the paracrine function of hMSCs. A heat map demonstrating overall differences in the secretion profiles of hMSCs cultured on different scaffolds (PCL, PCL/BG-WS, and PCL/BG-YS) measured on days 7 and 28. Results show total cytokine production (raw data not normalized to the DNA content) providing a bioengineering view on the global release of cytokines for each scaffold group.

CHAPTER 5

Elastin-like recombinamer coatings on composite scaffolds for the release of hypoxia-mimicking molecules enabling dual angiogenic and osteogenic stimulation for bone tissue engineering

Sophia Dalfino, Abshar Hasan, José Carlos Rodríguez-Cabello, Alvaro Mata, Gianluca Tartaglia, Claudia Dolci, Lorenzo Moroni

Submitted to Cell Biomaterials (Cell Press)

Abstract

Additive manufacturing (AM) techniques are widely used to produce scaffolds suited for mandible bone regeneration, aiming to replace autologous bone grafts as critical-sized defect treatment. Previous studies demonstrated that elastin-like recombinamers (ELR)-based coatings on AM scaffolds enable osteogenic differentiation and fast biomineralization of the scaffold surface *in vitro*. One major limitation remains the ability to simultaneously induce angiogenesis, which is essential for bone regeneration. Here, we hypothesized that 3D scaffolds with an ELR-based coating could be used as a drug-loading platform to release hypoxia-inducing small molecules. We fabricated polycaprolactone (PCL)/ β -tricalcium phosphates (β -TCP) scaffolds with ELR surface coatings, loaded with different concentrations of either deferoxamine (DFO) or 1,10-phenanthroline (Phen), to promote simultaneous pro-angiogenic and osteogenic processes. SEM images confirmed that a homogenous surface coating was achieved. In addition, the mineralization capacity of the coating remained functional, as organized needle-shaped mineralized structures were grown after incubation in a mineralization solution. The release profile of both Phen and DFO was determined by incubating the samples in physiological conditions. An initial burst release of approximately 96% and 45% of Phen and DFO, respectively, was measured in the first 24 h of incubation. A short exposure (i.e., 24 h) to a range of drug concentrations (DFO 1-100 μ M, Phen 2-200 μ M) was sufficient to induce HIF-1 α activation and VEGF upregulation in human mesenchymal stromal cells (hMSCs), cultured in 2D. Subsequently, hMSCs were seeded on ELR-coated scaffolds loaded with different Phen or DFO concentrations and cultured for 10 days. Gene expression profile displayed expression of HIF-1 α and VEGF genes in all conditions and significant upregulation of osteogenic-related genes RUNX2, ALP, BMP2 and SP7 for Phen 20 μ M and DFO 100 μ M. These results demonstrated the potential of our system in promoting simultaneous osteogenic and pro-angiogenic response and the interplay between these two processes.

Keywords

Bone Tissue Engineering, Elastin-like Recombinamers, Hypoxia, Angiogenesis, 3D Scaffolds

Introduction

The need for engineered bone grafts for the repair of craniomaxillofacial defects is increasing to overcome the limitations of the gold standard treatment, fibula flap transplantation [1]. In fact, this intervention is associated with several drawbacks, such as invasiveness, donor site morbidity, and low availability of the tissue that can be harvested [2]. In addition, the bone graft harvested from the fibula presents different anatomy compared to the mandible, causing morphological mismatches that increase the chances of failure and post-operative complications [3].

Additive manufacturing (AM) technologies, such as 3D-fiber deposition, offer the possibility to shape a variety of materials into 3D constructs, with control over architecture, pore geometry, as well as chemical and bioactive properties [4]. For bone-related applications, inorganic materials, such as tricalcium phosphates (TCP) or bioactive glasses, are typically blended with synthetic thermoplastic polymers, such as polycaprolactone (PCL) or polylactic acid, to fabricate AM composite scaffolds [5]. Various studies have demonstrated that such 3D constructs may support both osteogenic and angiogenic processes, depending on the composition of the inorganic component [6,7]. However, in these types of composite scaffolds, the bioactive component is initially hidden in the bulk of the fibers. The release of bioactive ions – involved in promoting cell differentiation – relies on the slow degradation of the thermoplastic polymers such as PCL. This results in a delayed cellular response, while the initial phase after implantation or culture is crucial in activating differentiation pathways in stem cells [8].

As a solution, several studies proposed surface modifications and coatings to promote an early biological response upon contact between scaffolds and cells. For example, polydopamine [9] or CaP coatings [10] on PCL scaffolds were shown to enhance cell adhesion, osteogenic differentiation, and bone formation both *in vitro* and *in vivo*. However, these coatings present limitations, such as non-specific binding properties of polydopamine, which could alter the intended bioactive effects and potentially trigger inflammatory responses [11], or the limited stability associated with fragile CaP-based coatings [12].

Recently, Hasan et al. reported the use of elastin-like recombinamers (ELRs)-based coatings on scaffold surfaces, which successfully supported osteogenesis and mineralization *in vitro* and *in vivo* [13]. ELR-based coatings represent a promising approach due to their versatility and tunability. ELRs are genetically engineered proteins, composed of repeating units of the VPGXG domain (V = valine, P = proline, G = glycine, X = any amino acid, except proline). The protein sequence can be engineered to incorporate specific domains to induce desired biological effects, such as promoting cell adhesion by including arginine, glycine, aspartic acid (RGD) peptide, or mineralization via a statherin domain (SNA15), known to have an affinity for Ca ions [14].

While promoting osteogenesis is essential, angiogenesis is equally critical in bone regeneration processes, as it facilitates nutrient and oxygen transport throughout the implanted constructs. One widely explored strategy to enhance angiogenesis is the fabrication of scaffold coatings to release pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF) [15]. However, the use of these molecules is often associated with short half-life and high costs [16]. As an alternative, small molecules with pro-angiogenic properties can be used, offering a more stable and cheap solution [17]. In addition, they can be rapidly taken up by the cells reaching the intracellular targets [17]. Small molecule compounds, such as deferoxamine (DFO), cobalt chloride or 1,10-phenanthroline (Phen), were shown to stabilize the transcription factor hypoxia-inducible factor 1- α (HIF-1 α) mimicking low oxygen environments [18,19]. The activation of HIF-1 α induces a cascade of pro-angiogenic events through the upregulation of vascular endothelial growth factor (VEGF) or other cytokines regulating angiogenesis [20].

In this study, we proposed the use of ELRs comprising a statherin-derived peptide sequence as a self-assembling mineralizing coating for PCL/ β -TCP composite scaffolds. Simultaneously, for the first time the ELR coating was used as a release platform for hypoxia-mimicking molecules, either DFO or Phen. We hypothesized that elastin-like recombinamer coatings incorporating hypoxia-mimicking small molecules would enable simultaneous activation of angiogenic and osteogenic pathways in human mesenchymal stromal cells, leading to improved bone-regenerative potential of the 3D scaffolds. We first validated and characterized the production of the scaffold coating and its functionality by assessing mineralization. Then, we screened the effect of different DFO or Phen concentrations on 2D cell culture of human mesenchymal stromal cells (hMSCs) and endothelial cells (hUVECs). hMSCs spreading and metabolic activity on ELR membranes were assessed. Finally, we investigated the osteogenic differentiation and hypoxic response of hMSCs seeded on the ELR-coated scaffolds loaded with different concentrations of each of the two molecules.

Materials and methods

Materials

All materials were purchased from Merck, unless stated otherwise. ELR with a statherin sequence (SN_A15) was purchased by Technical Proteins Nanobiotechnology (Valladolid, Spain). Polycaprolactone (PCL) and β -tricalcium phosphate (β -TCP) were provided by Corbion (Amsterdam, The Netherlands) and Kuros (The Netherlands), respectively.

Scaffold fabrication

Scaffolds were fabricated with a melt-extrusion printing system (Bioscaffolder, SYSENG, Germany), and they comprised PCL and β -TCP in a ratio 60:40. The composite material was prepared with a blending method described in previous work [6]. Briefly, PCL was dissolved in tetrahydrofuran solvent and mixed with TCP powders. Then, the material was precipitated in ethanol 100% v/v and dried under vacuum overnight. Scaffolds with a diamond-like biomimetic pore geometry were used for this study [6]. The material was extruded at $T=140^{\circ}\text{C}$, with a 25G nozzle, printing speed of $100\text{ mm}\cdot\text{min}^{-1}$, a pressure of 5 bar and a screw rotation of 11 rpm. Cylindrical scaffolds for cell culture experiments were fabricated with a diameter of 6 mm and a height of 2 mm.

ELR membranes fabrication and scaffold coating

The ELR membranes and the scaffold coating were produced by following a previously reported protocol [21]. The procedure was executed in low-humidity conditions ($< 20\%$) within an Aldrech® AtmosBag. Briefly, we started by dissolving the lyophilized ELR-statherin molecules in anhydrous DMF:DMSO (ratio 9:1) to obtain a 5% (w/v) ELR concentration. Then, an HDI crosslinker was added to the solution, at a 12:1 crosslinker to Lysine ratio. The solution was used to either fabricate membranes or to coat 3D scaffolds. For the membranes, glass coverslips (diameter = 9 mm) were placed in a custom made PDMS mold where 30 μl of ELR/HDI solution were drop-casted. The scaffolds were coated by dipping them in the ELR/HDI solution for approximately 15 seconds. Both membranes and scaffolds were left to dry for three days in the AtmosBag, washed with deionized water (dH_2O), and stored at 4°C .

ELR mineralization

A mineralization solution was prepared based on previously optimized methods [21]. First, nitric acid (69% v/v) was added to a 2 mM of hydroxyapatite powder and 2 mM of sodium fluoride in dH_2O solution until pH 3 was reached. Then, the pH was adjusted to 6 by adding ammonium hydroxide solution (30% v/v) dropwise. To validate the functionality of the ELR, the membranes and the coated scaffolds were incubated in the mineralization

solution at 37 °C for 7 days to induce mineralization. Finally, constructs were washed in dH₂O, dried and stored for further analysis.

ELR characterization

Scanning electron microscopy (SEM)

ELR membranes and scaffolds were coated with a Cressington sputter coater 108 Auto and imaged with SEM (Jeol JSM-IT200 InTouchScope), with a voltage of 10 kV and a working distance of 10 mm. The topography and coating distribution of the samples were qualitatively observed before and after mineralization to validate coating homogeneity and successful crystal formation.

Fourier Transform Infra-Red Spectroscopy (FTIR)

FTIR analysis was conducted (Nicolet iS50, Thermo Scientific) on ELR membranes before and after mineralization to characterize the chemical composition. The analysis was performed in the range of wavelength 4000-400 cm⁻¹, using 32 scans.

X-ray Diffraction (XRD)

XRD was used to assess mineral phase composition after mineralization of ELR membranes. The analysis was conducted in the range $5^{\circ} \leq 2\theta \leq 70^{\circ}$, with increments of 0.03°. The spectra were processed on Profex software (version 4.3).

Drug encapsulation and release

ELR 5% (w/v) solutions were prepared by dissolving different concentrations of two pro-angiogenic molecules, 1,10-phenanthroline (Phen, MW=180,21 g/mol) and deferoxamine (DFO, MW=560,684 g/mol). Drug-loaded ELR membranes and scaffold coatings were prepared following the same steps described in section 2.3.

Subsequently, the cumulative release profile of both molecules was quantified by incubating ELR-Phen/DFO membranes in PBS at 37°C. At different time points, the solution was collected and refreshed. UV-vis measurements (Cary 60, version 2.0) were conducted to quantify the drug concentration over time ($\lambda = 200-600$ nm). A standard curve was prepared for each drug in PBS.

Cell culture

Human mesenchymal stromal cells (hMSCs, PromoCell) were cultured in α -minimum essential medium (α -MEM) (Gibco) supplemented with 10% v/v fetal bovine serum (FBS) and 1% v/v ascorbic acid 2-phosphate (ASAP). The medium was refreshed every 2-3 days until reaching 80% confluency. Passage 5 cells were used for the experiments.

Human umbilical vein endothelial cells (HUVECs, Lonza) were maintained in endothelial cell growth medium ready to use kit (Promocell). The medium was refreshed every 2-3 days until reaching 80% confluency. Cells were used at passage 4-6 for the experiments.

2D drug screening

As the first biological test, hMSCs or HUVECs were seeded in treated 96-well plates at 10,000 cell/cm² density and cultured until they reached 80% confluency. Then, different Phen or DFO concentrations were dissolved in culture medium (**Table 1**) and incubated with either hMSCs or HUVECs for 24 h to assess cytotoxicity. Cell morphology was qualitatively evaluated through brightfield imaging.

Table 1. Drug concentrations tested in 2D screening on hMSCs and HUVECs.

Drug	Concentrations
DFO	1 mM, 100 μ M, 10 μ M, 1 μ M
Phen	2 mM, 200 μ M, 20 μ M, 2 μ M

Cell seeding on the ELR membranes

ELR membranes loaded with different drug concentrations (**Table 2**) were produced and sterilized with UV for 5 min. The sterile membranes were placed in a non-treated 48-well plate and held in place with O-rings. hMSCs were seeded on the membranes at a density of 50,000 cells/membrane in 300 μ l of cell culture medium.

Table 2. Drug concentrations tested in ELR membranes and ELR-coated scaffolds seeded with hMSCs.

Drug	Concentrations	Sample name
DFO	200 μ M, 20 μ M, 2 μ M	P200, P20, P2
Phen	100 μ M, 10 μ M, 1 μ M	DFO100, DFO10, DFO1

Cell seeding on the ELR scaffolds

ELR-coated PCL/ β -TCP scaffolds loaded with different Phen or DFO concentrations (**Table 2**) were produced and sterilized with UV for 5 min. The scaffolds were placed in a non-treated 48-well plate. hMSCs were seeded following a dextran seeding method at a density of 500,000 cells/scaffold in a 30 μ l seeding volume. After allowing cell attachment for 4 h, cell culture medium supplemented with 1% v/v Penicillin-Streptomycin was added to the wells. Scaffolds were cultured for 10 days, and the medium refreshed every 2-3 days.

Biological evaluation

Metabolic activity

Metabolic activity was assessed on different time points using a Presto Blue® Cell viability Reagent (**Table 3**). Briefly, a 10% v/v PrestoBlue solution in culture medium was incubated for 20 minutes (for 2D and membranes) or 45 minutes (for the scaffolds) at 37°C. Then, 100 µl of solution in duplicates were collected and transferred to a black 96-well plate. Samples were washed with PBS and fresh medium was added to the wells. The fluorescent signal was recorded with a plate reader at excitation/emission wavelength 535-615 nm. Cells without drug were used as control.

Table 3. Summary of the time points on which the metabolic activity was evaluated for the different biological experiments.

Experiment	Time points
2D drug screening	24h
Seeding on the ELR membranes	1, 3, 7 days
Seeding on the scaffolds	3, 7, 10 days

Immunofluorescence

At different time points, cells were fixed with 4% v/v paraformaldehyde (PFA) for 10 minutes (2D and membranes) or 20 minutes (scaffolds) and washed with PBS. For immunostaining, first Triton-X 0.1% v/v was incubated for 10 min to allow permeabilization, followed by a blocking step with BSA 3% w/v for 1.5 h. Subsequently, primary antibodies and phalloidin were incubated overnight at 4°C. Then, the samples were washed, incubated with secondary antibody (1:300) for 45 min, and washed again before adding DAPI (1:300) for 20 min. Fixable live/dead staining (Invitrogen, Far red) was performed according to manufacturer's instructions to assess cell viability on the ELR membranes. Samples were imaged with a Slide Scanner microscope (Nikon Ti-E microscope, equipped with a Lumencor Spectra light source, an Andor Zyla 5.5 sCMOS camera, a Nikon DS-Ri2 color camera, and an MCL NANO Z200-N TI z-stage) or with Leica TCS SP8 CARS confocal microscope. **Table 4** summarizes the staining that were performed for each experimental condition.

Table 4. Summary of the staining conditions for the different biological experiments.

Experiment	Time point	Antibodies
2D drug screening	24h	Phalloidin (Alexa Fluor 647, Thermofisher, 1:500) hMSCs: HIF- α (Mouse, Abcam ab279654, 1:400) hUEVCs: CD31 (Mouse, Abcam ab24590, 1:400)
Seeding on the ELR membranes	Day 1	Fixable live/dead (Invitrogen)
	Day 7	Phalloidin (Alexa Fluor 647, Thermofisher, 1:500)
Seeding on the scaffolds	Day 10	Phalloidin (Alexa Fluor 647, Thermofisher, 1:100) HIF- α (Mouse, Abcam ab279654, 1:300) RUNX2 (Rabbit, Abcam ab192256, 1:300)

Gene expression

Gene expressions of relevant angiogenic and endothelial genes were evaluated through qPCR (**Table 5**). At different time points, cells were washed with PBS, collected in 1 ml Trizol (Invitrogen) and stored at -80°C. RNA was extracted with chloroform-isopropanol method, and its concentration was quantified with a BioDrop μ LITE instrument (Scientific Laboratory Supplies). iScript cDNA Synthesis Kit (Bio-Rad) was used for cDNA synthesis according to manufacturer's instructions. qPCR reaction was performed in 10 μ l volume, mixing 4 ng of cDNA with iQ SYBER green Supermix (Bio-Rad) and primer solution. Relative gene expression was calculated with the $2^{-\Delta\Delta C_t}$ method. Values were normalized against untreated samples.

Table 5. List of genes and primers analyzed with qPCR performed on hMSCs and hUVECs cultured in 2D or on the scaffolds for 24h or 10 days.

Gene	Forward sequence	Reverse sequence
B2M (Housekeeping)	ACAAAGTCACATGGTTCACA	GACTTGTCTTTCAGCAAGGA
2D drug screening (hMSCs and hUVECs)		
HIF- α	TGCAACATGGAAGGTATTGC	TTCACAAATCAGCACCAAGC
VEGFA	ATCTTCAAGCCATCCTGTGTGC	GCTCACCGCCTCGGCTTGT
PECAM1	GAAAGCCAAGGCCAAGCAGATG	TTCCACGGCATCAGGGACAG
KDR	CCCTACAAGACCAAAGGGGCAC	GCGATGCCAAGAACTCCATGC
Seeding on the scaffolds (hMSCs)		
HIF- α	TGCAACATGGAAGGTATTGC	TTCACAAATCAGCACCAAGC
VEGF-A	ATCTTCAAGCCATCCTGTGTGC	GCTCACCGCCTCGGCTTGT
RUNX2	TCAACGATCTGAGATTTGTGGG	GGGGAGGATTTGTGAAGACGG
ALP	ACAAGCACTCCCACTTCATC	TTCAGCTCGTACTGCATGTC
BMP2	AGCCCATAGTGCTTGTAAGG	GCATCTGTTCTCGGAAAACCT
SP7	CCTCTGCGGGACTCAACAAC	AGCCCATAGTGCTTGTAAGG

Statistical analysis

All data are presented as mean $\pm$ standard deviation. Statistical analysis was done on GraphPad Prism (version 10). Data normality was assessed with Shapiro-Wilk test. When normal, statistical differences were tested with parametric One-way ANOVA. Non-parametric tests were used otherwise. Statistical significance represented by: (****) $p < 0.0001$, (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$.

Results and discussion

ELR coating characterization and mineralization

We selected an ELR sequence containing a statherin-derived peptide, because this motif has high affinity for calcium ions, facilitating localized precipitation of minerals on the surface of the coating. Compared to other ELR molecules, ELR-statherin coatings induced the formation of a higher amount of mineralized structures exhibiting organized

nanocrystals when immersed in a mineralization solution [22]. In addition, ELR coatings containing statherin have been previously demonstrated to significantly enhance osteogenic differentiation and matrix mineralization in hMSCs, outperforming other ELR sequences lacking this domain [23,24].

Drop-casted ELR membranes and ELR-coated PCL/ β -TCP scaffolds were produced. The samples were imaged with SEM, which confirmed the successful formation of the ELR membranes (**Figure S1A**) and scaffold coatings (**Figure 1A**). The ELR coating was homogeneously distributed over the scaffold surface. The formation of typical ELR spherulites comprising aligned apatite nanocrystals was observed on the coating (**Figure S1A**). As previously reported by Elsharkawy et al., these supramolecular structures result from protein conformational rearrangements and from the modulation of disordered-ordered regions within the protein sequence, controlled by crosslinking conditions [22].

ELR spherulites are essential to initiate mineral nucleation and subsequently induce the organized biomineralization processes. We validated the functionality of the ELR coating by immersing membranes and scaffolds in a mineralization solution. After 7 days of incubation, a uniform deposition of needle-shaped crystals was identified on the membranes (**Figure S1B**) and on the surfaces of the scaffold fibers (**Figure 1B**). EDX analysis showed that the composition of the crystals contained predominantly Ca, P and F ions (**Figure 1C, Figure S1**). Phosphate groups (PO_3^{4-}) were identified after mineralization in the FTIR spectra, at wavelengths 562 and 1016 cm^{-1} (**Figure 1D**). Notably, the amide I and III peaks could only be observed in the non-mineralized ELR membranes (wavelengths 1624 and 1246 cm^{-1}), implying that mineralization induced full coverage of the surface. Finally, XRD analysis indicated that the deposited mineral phase corresponded to fluorapatite (**Figure 1E**). We attributed the chemical composition of the mineralized surface to the presence of hydroxyapatite and sodium fluoride in the mineralization solution.

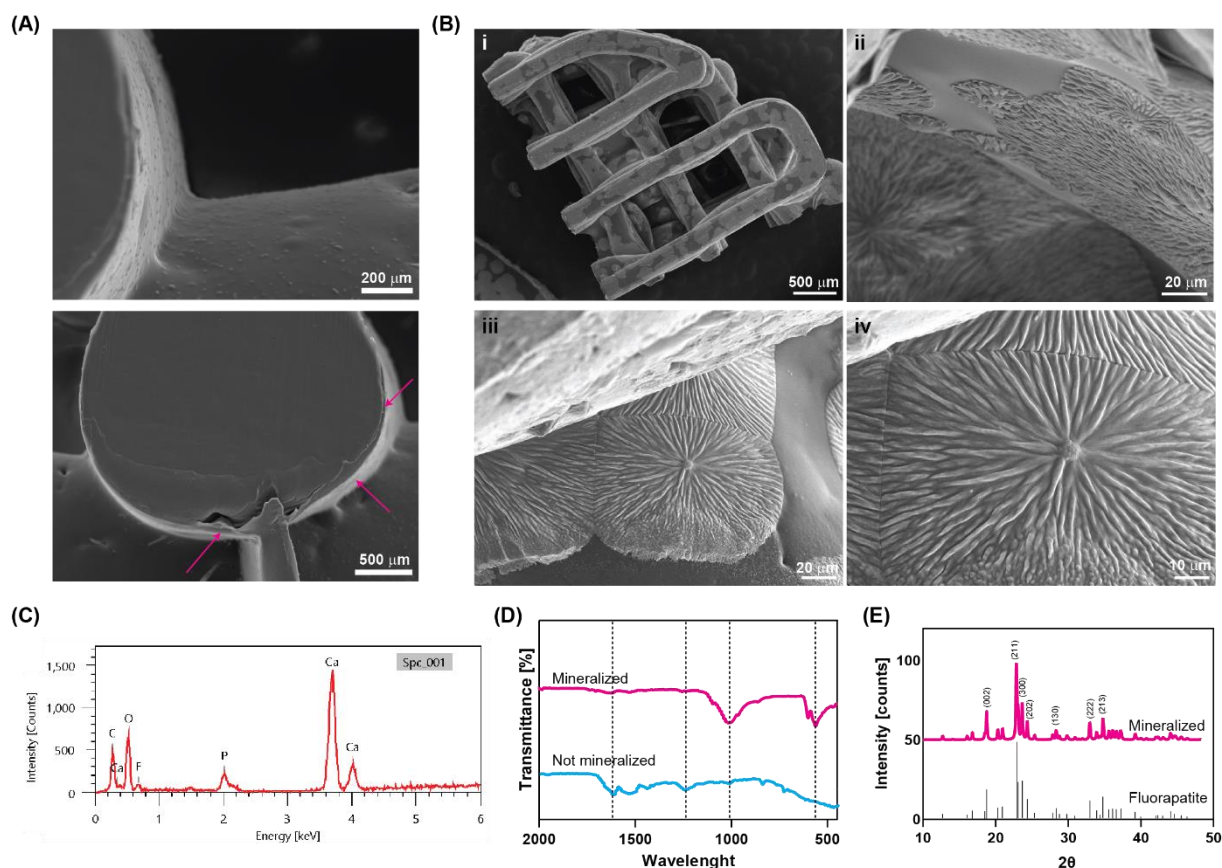


Figure 1. Characterization of the ELR coating on PCL/ β -TCP scaffold fibers. (A) SEM images of the ELR coating on the fibers, displaying homogenous distribution. The pink arrows indicate the layer of coating covering the fiber surface. (B) SEM images of the ELR coating on the scaffolds after 7 days of mineralization, showing the formation of typical needle-shaped crystals, at increasing magnification: *i*) scale bar = 500 μ m, *ii*) scale bar = 20 μ m, *iii*) scale bar = 20 μ m, *iv*) scale bar = 10 μ m. (C) EDX analysis of mineralized ELR coating indicating the presence of Ca, P and F ions in the crystals. (D) Zoom of the FTIR peaks in a range 2000-500 cm^{-1} before (blue) and after (pink) mineralization of the ELR coating. Amide peaks can be observed before mineralization at around 1700 and 1200 cm^{-1} ; phosphate group peaks appear after mineralization at around 1000 and 600 cm^{-1} . (E) XRD spectra of mineralized ELR, displaying the characteristic fluorapatite peaks.

Phen and DFO release profile from ELR membranes

Two pro-angiogenic molecules were loaded in the ELR coating; 1,10-phenanthroline (Phen, 200 μ M) and deferoxamine (DFO, 100 μ M) (**Figure 2A**). Both molecules were reported to have hypoxia-mimicking function, by inhibiting the prolyl hydroxylase (PHD) activity, leading to stabilization and activation of the HIF-1 α factor [25–28]. After encapsulation, the loaded ELR membranes were incubated in physiological conditions, and the molecule release was measured over 14 days (**Figure 2B**). The cumulative release profile of both Phen and DFO exhibited a rapid initial phase during which approximately $96.33 \pm 3.17\%$ and $42.55 \pm 8.56\%$ of their respective content were released within the first 24h of incubation. A burst release was also observed in other studies when small molecules were not covalently entrapped or loaded into nanocarriers. For instance, Rassu et al. presented chitosan/alginate hydrogels loaded with DFO reporting a burst release of 46% when the drug was directly dispersed in the hydrogel [29]. In the second phase, the

release stabilized into a plateau at approximately 100% for Phen and 80% for DFO (**Figure 2B**). We hypothesized that the differences in release profile resulted from either the five times larger size of DFO or (partial) attachment due to a reaction between the free amine and the HDI crosslinker.

The induction of early hypoxic triggers has been known to induce early angiogenic cascades [26,30–32]. Since Phen was almost fully released within 24h, we focused on evaluating the early effects of the small molecules on angiogenic pathways before performing a 10-day study on 3D scaffolds.

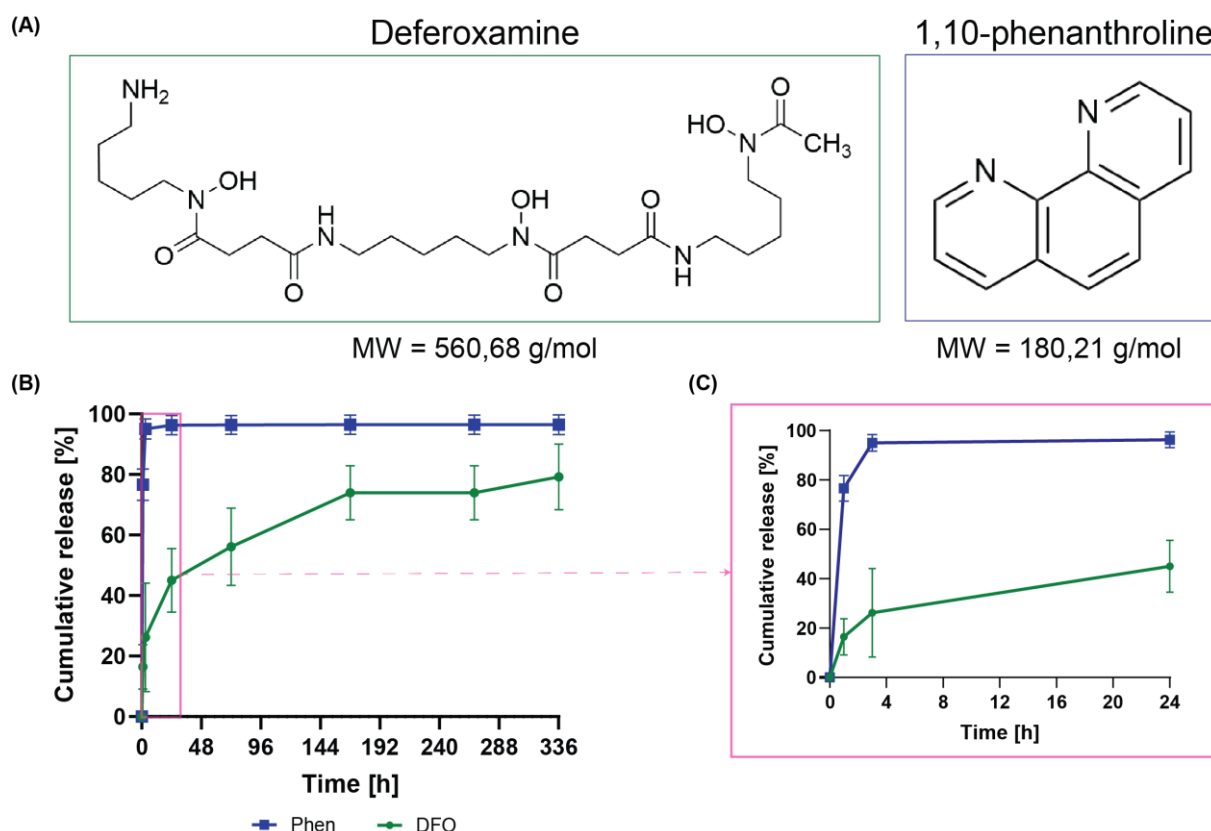


Figure 2. Release study of the encapsulated Phen and DFO from ELR membranes. (A) Chemical structure and molecular weight of Deferoxamine (DFO) and 1,10-phenanthroline (Phen). (B) Phen and DFO release from ELR coating over 14 days of incubation in PBS and (C) Zoom in on Phen and DFO release in the first 24h of incubation in PBS, showing burst release of the drugs.

Cell behavior in 2D when exposed to Phen or DFO

To assess whether the burst release of DFO and Phen could effectively activate hypoxic response within a short time window, we performed a first biological evaluation in a 2D cell culture model, where hMSCs and hUEVCs were exposed to decreasing Phen (200 – 2 μ M) and DFO (100 – 1 μ M) concentrations for 24h. The screening was conducted on both cell types due to their involvement in bone regenerative processes *in vivo*.

Cellular response to the drugs was determined based on morphological changes and metabolic activity. From brightfield images we observed that both hMSCs and hUEVCs

presented a round morphology when exposed to the highest Phen and DFO concentrations (Phen = 2mM and DFO = 1 mM) (**Figure 3A, Figure S2A**). In addition, the lowest metabolic activity was observed in these conditions (**Figure 3B, Figure S2B**). These data suggest cell death and these concentrations were excluded from further analysis. In all the other conditions, cells displayed elongated morphology and high levels of metabolic activity (**Figure 3A-B, Figure S2A-B**). Both hMSCs and hUVECs exhibited concentration-dependent behavior in response to Phen, with lower metabolic activity when the drug concentration was increased.

Subsequently, we evaluated the effect of both drugs at a gene expression level after 24h incubation at different concentrations (**Figure 3C-D, Figure S2**). hMSCs expressed HIF-1 α in all the tested conditions (**Figure 3C**). No significant differences were observed in the DFO group, whereas the Phen group displayed a significant downregulation of the gene for Phen200 and Phen20 (0.09 ± 0.05 and 0.17 ± 0.04 for Phen200 and 20, respectively). Concerning VEGF expression, an opposite trend was recorded, where Phen200, Phen20 and DFO100 induced significant upregulation of the gene of around 36-, 33-fold and 3.5-fold, respectively, compared to control condition (**Figure 3D**).

These results suggest that an earlier upregulation of HIF-1 α may have occurred in the Phen200, Phen20 and DFO100 conditions, triggering an upregulation of VEGF as a downstream target [33–35]. We speculated that the observed downregulation of HIF-1 α in Phen200 and Phen20 after 24h may result from a feedback mechanism, where mRNA expression is being suppressed while the protein activity persists at the post-transcriptional level, as reported before [36]. Interestingly, this pattern was only observed in the Phen group, which could be attributed to a higher cellular stress induced by the high Phen concentrations, as also suggested by the lower metabolic activity compared with DFO.

Immunofluorescence images were taken to evaluate specific markers in response to different drug concentrations (**Figure S3**). We observed the presence of HIF-1 α in hMSCs for all drug concentrations (**Figure S3**). However, only Phen200 and Phen20 displayed nuclear translocation of the marker, indicative of its activation, further supporting what observed at a gene expression level (**Figure 3D**). We concluded that 24h of drug exposure was already effectively inducing the activation of the HIF-1 α pathway for Phen200, Phen20 and DFO100 conditions.

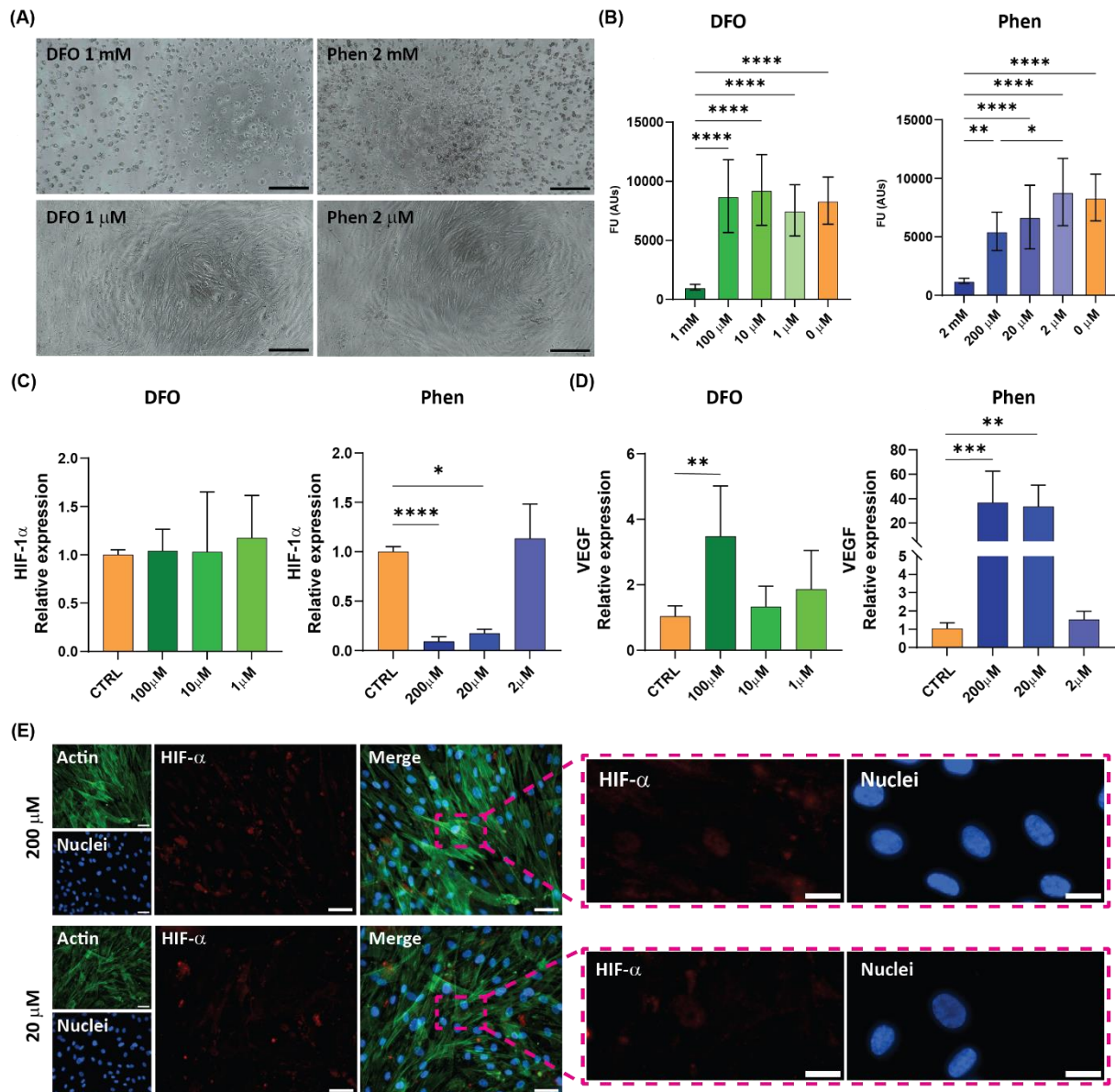


Figure 3. Evaluation of hMSCs response after 24h incubation to different concentrations of Phen or DFO. (A) Representative brightfield images of hMSCs exposed to the highest or lowest Phen and DFO concentration for 24h. Scale bar = 200 μ m. (B) Metabolic activity of hMSCs after 24h of exposure to different DFO and Phen concentrations. (C) HIF-1 α and (D) VEGF gene expression in hMSCs after 24h of incubation with different Phen and DFO concentrations. (E) Representative immunostaining images of hMSCs incubated for 24h with Phen200 and Phen20, showing nuclear localization of HIF-alpha (red), F-actin (green) and nuclei (blue). Scale bar = 200 μ m. Statistical significance represented by: ****<0.0001, ***<0.001, **<0.01, *<0.05.

Cell adhesion and metabolic activity on ELR membranes

After demonstrating the effectiveness of the small molecules in the first 24h, we proceeded to a proof-of-concept experiment where we evaluated the response of hMSCs when directly seeded on ELR membranes. We loaded different concentrations of either Phen (200 μ M, 20 μ M, 2 μ M) or DFO (100 μ M, 10 μ M, 1 μ M) in the membranes.

On day 1, we assessed cellular morphology and viability through DAPI/Phalloidin and dead staining, respectively. In all conditions, we observed initially poor cell attachment,

with formation of spheroid-like aggregates (**Figure 4A**). We attributed this behavior to low hydrophilicity of the ELR membranes, which was already reported in a previous study [13]. Despite the initial low adhesion, hMSCs showed increased metabolic activity over 7 days (**Figure 4B**), with the DFO groups displaying higher values compared to both the untreated control and the Phen group. Fluorescent images showed full population of the membranes area in all drug-loaded conditions (**Figure 4C, Figure S4**).

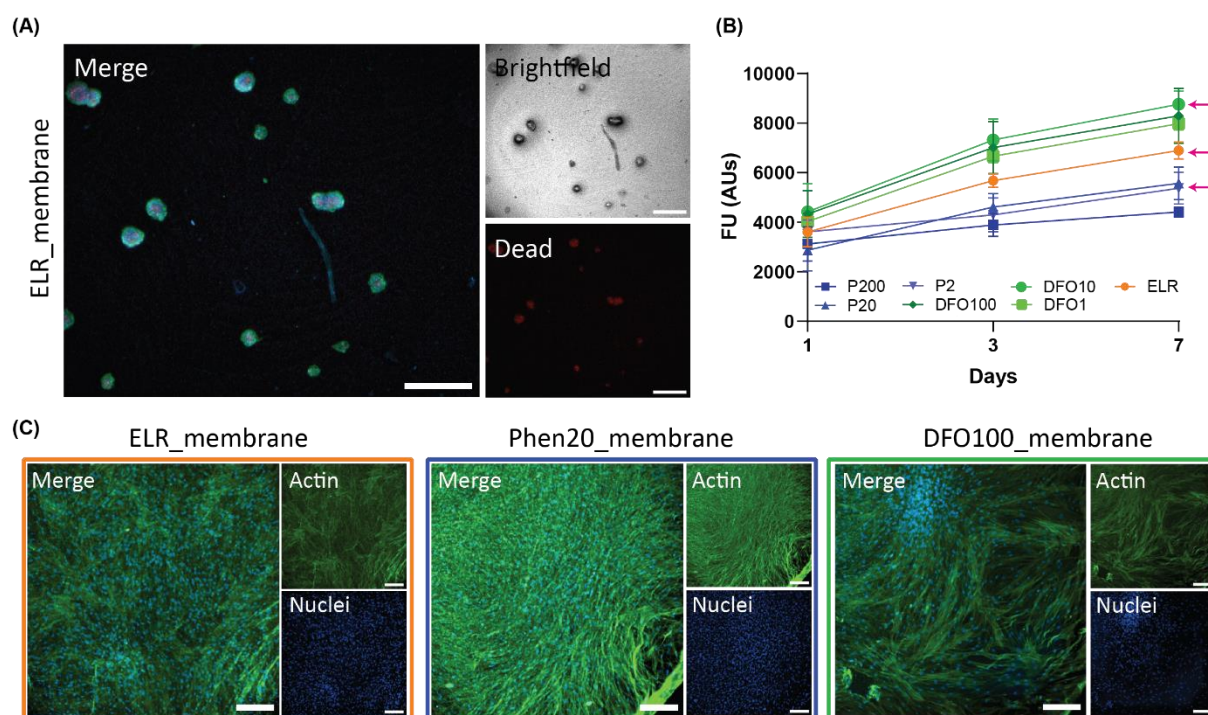


Figure 4. Evaluation of hMSCs when seeded on ELR membranes loaded with different Phen or DFO concentrations. (A) Representative fluorescent and brightfield image of ELR only membranes, displaying dead cells (red), nuclei (blue) and F-actin (green) of hMSCs. Scale bar = 200 μ m. (B) Metabolic activity of hMSCs over 7 days in response to different drug concentrations. The arrows indicate (from top to bottom): DFO10, ELR only, and Phen20, which resulted to be the two best conditions of each group in comparison with the untreated control. (C) Representative fluorescent images of ELR only, ELR-Phen20 and ELR-DFO100 membranes with seeded hMSCs, showing cell morphology (nuclei-blue, F-actin-green) after 7 days of culture. Scale bar = 200 μ m.

Pro-angiogenic and osteogenic response to ELR-coated scaffolds

Finally, we evaluated the capacity of ELR coated 3D composite scaffolds (PCL/ β -TCP) releasing decreasing concentrations of either Phen (200 - 2 μ M) or DFO (100 - 1 μ M) to induce a pro-angiogenic response and osteogenic commitment on hMSCs after 10 days of culture. Cells presented increasing metabolic activity for all the conditions. As previously observed (**Figure 3B**), Phen200 and Phen20 scaffolds displayed the lowest metabolic activity values (**Figure S5A**).

Gene expressions were investigated in response to the different drug conditions. We first assessed pro-angiogenic markers. Minor differences among conditions were observed in the expression of HIF-1 α with only around 0.7-fold downregulation of HIF- α for Phen2

samples compared to ELR control (**Figure 5A**). The expression of HIF-1 α downstream genes VEGF, EPO, ANGPTL and IL-8 was also assessed (**Figure 5A, Figure S5B**). An increasing trend was observed for the Phen20 condition in the expression of EPO and ANGPTL. On the other hand, DFO group displayed a significant upregulation of EPO for the DFO1 condition, while no differences were observed for VEGF and ANGPTL genes. We hypothesized that an upregulation of these markers occurred at earlier time points in response to the initial burst release of the drugs. Regarding the expression of IL-8, both Phen and DFO groups showed a significant upregulation of the gene at the highest drug concentrations (i.e., Phen200 and DFO100) (**Figure S5B**). Hypoxia-mediated activation of IL-8 was previously reported as an indirect consequence of HIF-1 α upregulation through the NF- κ B pathway [37,38]. Additionally, IL-8 is also known to be a pro-inflammatory factor, thus we cannot exclude that its upregulation at the highest drug concentrations might also be explained by activation of inflammatory response [26].

Next, we evaluated the expression of osteogenic markers by comparing both Phen and DFO groups to ELR only scaffolds. Phen20 scaffolds promoted a significant upregulation of ALP, BMP2 and SP7, with around 2-, 2.5- and 1.5-fold increase, respectively (**Figure 5A**). BMP2 was also 2-fold upregulated in the DFO100 condition. On the contrary, early osteogenic markers RUNX2 and ALP were approximately 0.5-fold downregulated. The different trends in gene upregulation observed by comparing Phen and DFO group could be attributed to a different timing in the osteogenic pathway, also related to different molecule release profiles. Additionally, when comparing Phen and DFO patterns, we observed that Phen induced the upregulation of osteogenic markers at lower concentrations, whereas DFO required the highest concentration to elicit similar effects. This difference may confirm the stronger hypoxia-mimicking effect of Phen, as previously reported [26]. Immunostaining confirmed the presence of RUNX2 and HIF-1 α (**Figure 5B-C**) in all the samples, with no qualitative differences among conditions. RUNX2 was mostly found in cell nuclei, while HIF-1 α localization was mostly cytoplasmic, aligning with gene expression results.

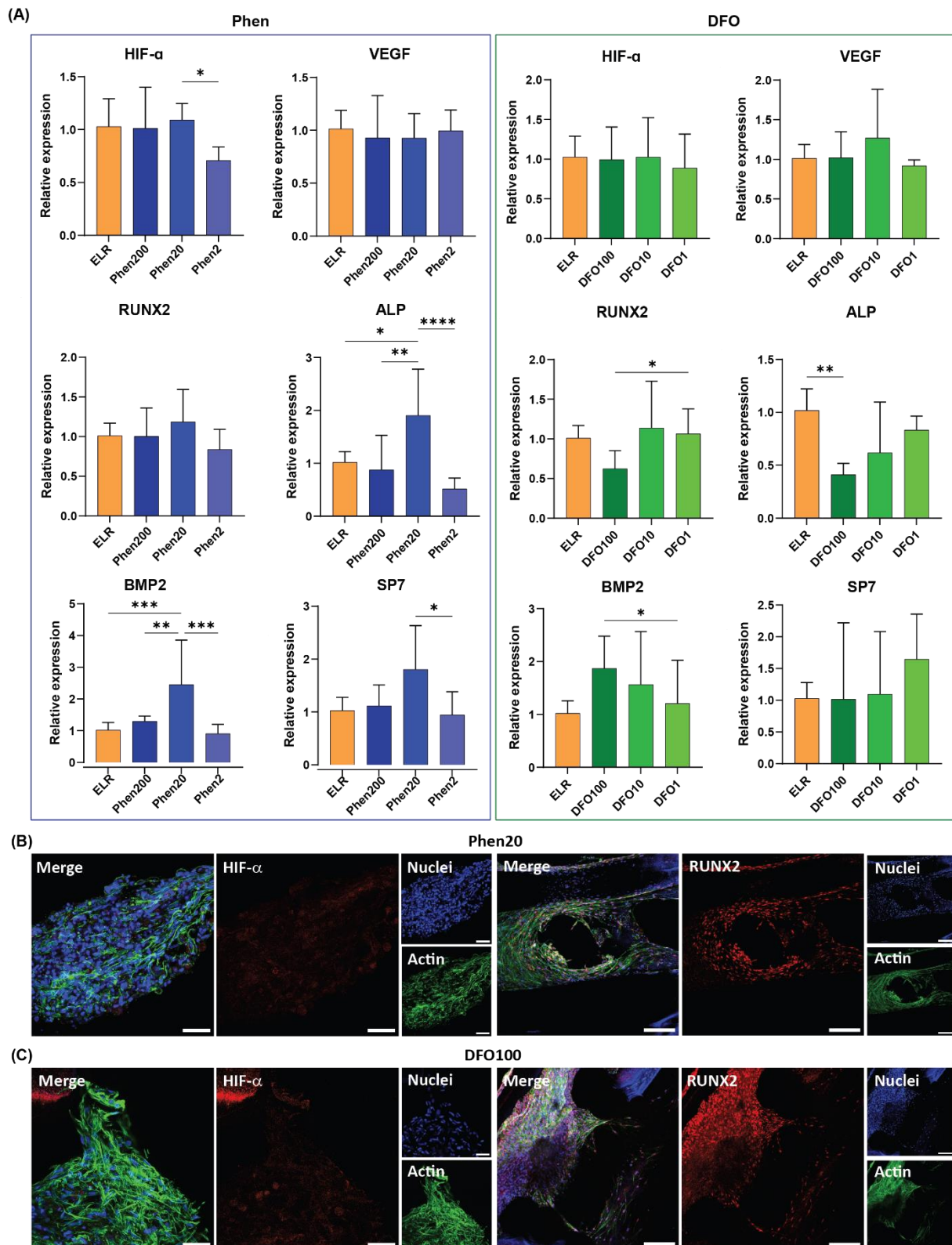


Figure 5. Evaluation of hMSCs response to the ELR-coated scaffolds at different drug concentrations. (A) Gene expression of hMSCs of pro-angiogenic and osteogenic genes after 10 days of culture. (B) Representative immunostaining image (left) of ELR-Phen20 scaffolds after 10 days of culture showing the cytoplasmic localization of HIF-alpha (red), nuclei (blue) and F-actin (green). Scale bar = 50 μ m. Representative immunostaining image (right) of ELR-Phen20 scaffolds after 10 days of culture showing the nuclear localization of RUNX2 (red), nuclei (blue) and F-actin (green). Scale bar = 200 μ m. (C) Representative immunostaining image (left) of ELR-DFO100 scaffolds after 10 days of culture

showing the cytoplasmic localization of HIF- α (red), nuclei (blue) and F-actin (green). Scale bar = 50 μ m. Representative immunostaining image (right) of ELR-DFO100 scaffolds after 10 days of culture showing the nuclear localization of RUNX2 (red), nuclei (blue) and F-actin (green). Scale bar = 200 μ m. Statistical significance represented by: ****<0.0001, ***<0.001, **<0.01, *<0.05.

Our findings demonstrate that the induction of a hypoxia-mimetic environment through the release of small molecules from 3D scaffolds resulted in a synergistic effect where also osteogenic differentiation was enhanced. We hypothesized the presence of a temporal pattern characterized by early HIF-1 α activation and VEGF upregulation within 24 h followed by increased expression of osteogenic markers such as ALP, BMP2, and SP7 after 10 days. This suggests a sequential interplay between angiogenic and osteogenic signaling. Transient stabilization of HIF-1 α stimulates VEGF release, which not only promotes endothelial activation but can also indirectly enhance osteogenic differentiation of mesenchymal stromal cells through paracrine and autocrine mechanisms [39]. In particular, VEGF has been shown to interact with the RUNX2 pathway and to activate downstream PI3K/AKT/mTOR signaling, thereby coupling angiogenesis and osteogenesis during bone formation [39,40]. This mechanistic cascade may therefore explain the dual pro-angiogenic and osteogenic responses observed in our ELR-based scaffolds loaded with hypoxia-mimicking molecules. The interplay between hypoxic and osteogenic pathways in bone formation processes is well documented [41–44]. Indeed, several 2D studies reported that exposing cells (e.g. hMSCs or adipose-derived stem cells) to hypoxic conditions *in vitro* or *in vivo* enhanced expression of osteogenic genes (e.g. ALP, OCN, RUNX2, COLL1, BMP2), bone formation and angiogenesis [45,46].

With the dual purpose of enhancing osteogenesis and angiogenesis, other groups proposed scaffold-based systems to deliver hypoxic molecules or ions [47–49]. To the best of our knowledge, no studies reported on the release of Phen from osteogenic scaffolds. Despite its well-documented hypoxia-mimicking properties, the use of Phen has been limited to 2D cultures, drug delivery system applications [50] or as injectable molecules in combination with ceramic discs [51]. Our study demonstrates for the first time that Phen can be effectively incorporated into a scaffold coating, acting as a potent molecule to simultaneously enhance angiogenic and osteogenic responses.

On the other hand, most studies focused on delivery of DFO through different strategies [52–54]. For example, some groups have described polymer or metal 3D scaffolds coated with GelMA-based hydrogels loaded with DFO with or without additional nanocarriers, showing increased early expression of hypoxia markers (e.g. HIF-1 α or VEGF) and later osteogenic differentiation and/or *in vivo* bone formation [55,56]. Here, a sustained release of the drug was achieved, with a significant biological effect visible within the first 1-3 days of incubation [56], aligning with the findings of our study. Yao et al. reported a gelatin nanofibrous scaffold where, differently from our approach, DFO was covalently grafted onto the surface, inducing upregulation of VEGF expression *in vitro* and superior bone formation

in vivo [57]. Notably, the authors demonstrated that covalently grafted DFO displayed higher iron-binding capacity compared to physically adsorbed DFO, demonstrating a higher efficiency of the grafted system.

In this study, both DFO and Phen were selected as model hypoxia-mimicking molecules because they stabilize HIF-1 α through PHD inactivation. DFO is a clinically established agent commonly used in tissue engineering, whereas Phen represents a less explored, smaller, and more stable alternative. Testing both compounds allowed us to evaluate the versatility of the ELR-based coating as a drug-delivery platform and to compare their relative abilities to induce angiogenic and osteogenic responses. Future studies could further investigate the mechanism of DFO and Phen encapsulation and release in our system (see section 3.2), assessing whether (partial) grafting to the ELR coating or loading into nanocarriers could increase their pro-angiogenic effect. In addition, to further enhance scaffold's osteogenic properties, future research could add a pre-mineralization step to the ELR coating, prior to *in vitro* experiments. Extended cell culture studies (e.g. up to 28 days) would be also needed to assess the effect on extracellular matrix maturation. Finally, implementing a direct co-culture of hMSCs and endothelial cells would provide deeper insights into the scaffold's capacity to support vascularization.

Conclusion

In this study, we demonstrated that 3D composite PCL/ β -TCP scaffolds coated with ELR loaded with two hypoxia-mimicking molecules, either Phen or DFO, could effectively support simultaneous mineralization as well as pro-angiogenic and osteogenic effects in hMSCs. Our data showed that rapid molecule release stimulated the expression of pro-angiogenic genes and upregulation of osteogenic markers under specific molecule concentrations. These findings support the idea that our system represents a promising strategy for enhancing angiogenic and osteogenic processes during bone regeneration. Future *in vivo* validation would be important to confirm the relevance of this approach in promoting bone regeneration for future clinical translation.

Acknowledgments

The authors acknowledge the NIHR Nottingham Biomedical Research Centre at University of Nottingham, Nottingham, UK and Project AOCMF-21-04S from the AO Foundation, AO CMF. The AO CMF is a clinical division of the AO Foundation — an independent medically guided not-for-profit organization. We are grateful for funding from the Spanish Government (grant PID2022-137484OB-I00 funded by MCIN/AEI/10.13039/501100011033 and by ERDF, EU), from the Department of Education, *Junta de Castilla y León* (grant VA188P23 and CLU-2023-1-05 cofunded by ERDF, EU) and from *Centro en Red de Medicina Regenerativa y Terapia Celular de Castilla y León*.

Declaration of interests

Prof. Dr. Lorenzo Moroni is member of the journal's advisory board. José Carlos Rodríguez Cabello is scientific advisor and co-founder of Technical Proteins Nanobiotechnology.

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CHAPTER 5

Supplementary information

Elastin-like recombinamer coatings on composite scaffolds for the release of hypoxia-mimicking molecules enabling dual angiogenic and osteogenic stimulation for bone tissue engineering

Sophia Dalfino, Abshar Hasan, José Carlos Rodríguez-Cabello, Alvaro Mata, Gianluca Tartaglia, Claudia Dolci, Lorenzo Moroni

Submitted to Cell Biomaterials (Cell Press)

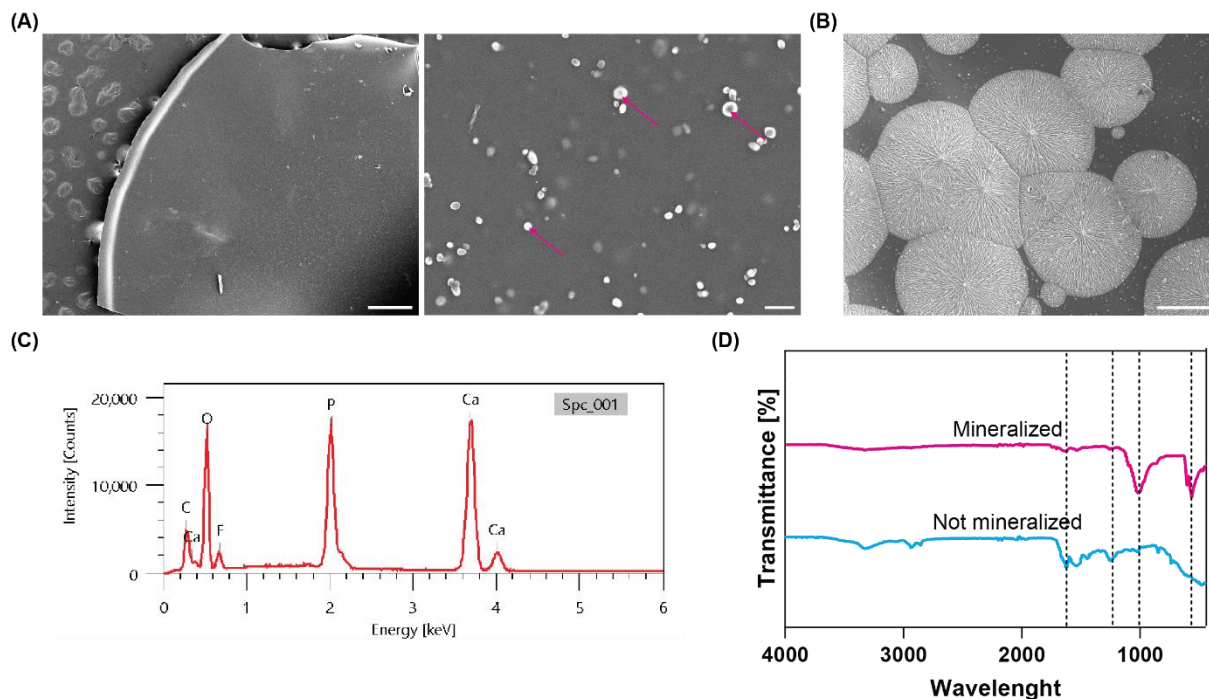


Figure S1. Characterization of the ELR membranes. (A) SEM images of the ELR membranes. Scale bar = 500 μm (left) and 10 μm (right). Pink arrows indicate the spherulites that form on the membranes after crosslinking. (B) SEM images of the ELR membranes after 7 days of mineralization, showing the formation of typical fluorapatite crystals. Scale bar = 100 μm . (C) EDX analysis displays the presence of Ca, P and F ions in the crystals. (D) Full FTIR peaks in a range 4000-400 cm^{-1} before and after mineralization of ELR coating. Amide peaks can be observed before mineralization at around 1700 and 1200 cm^{-1} ; phosphate group peaks show after mineralization at around 1000 and 600 cm^{-1} .

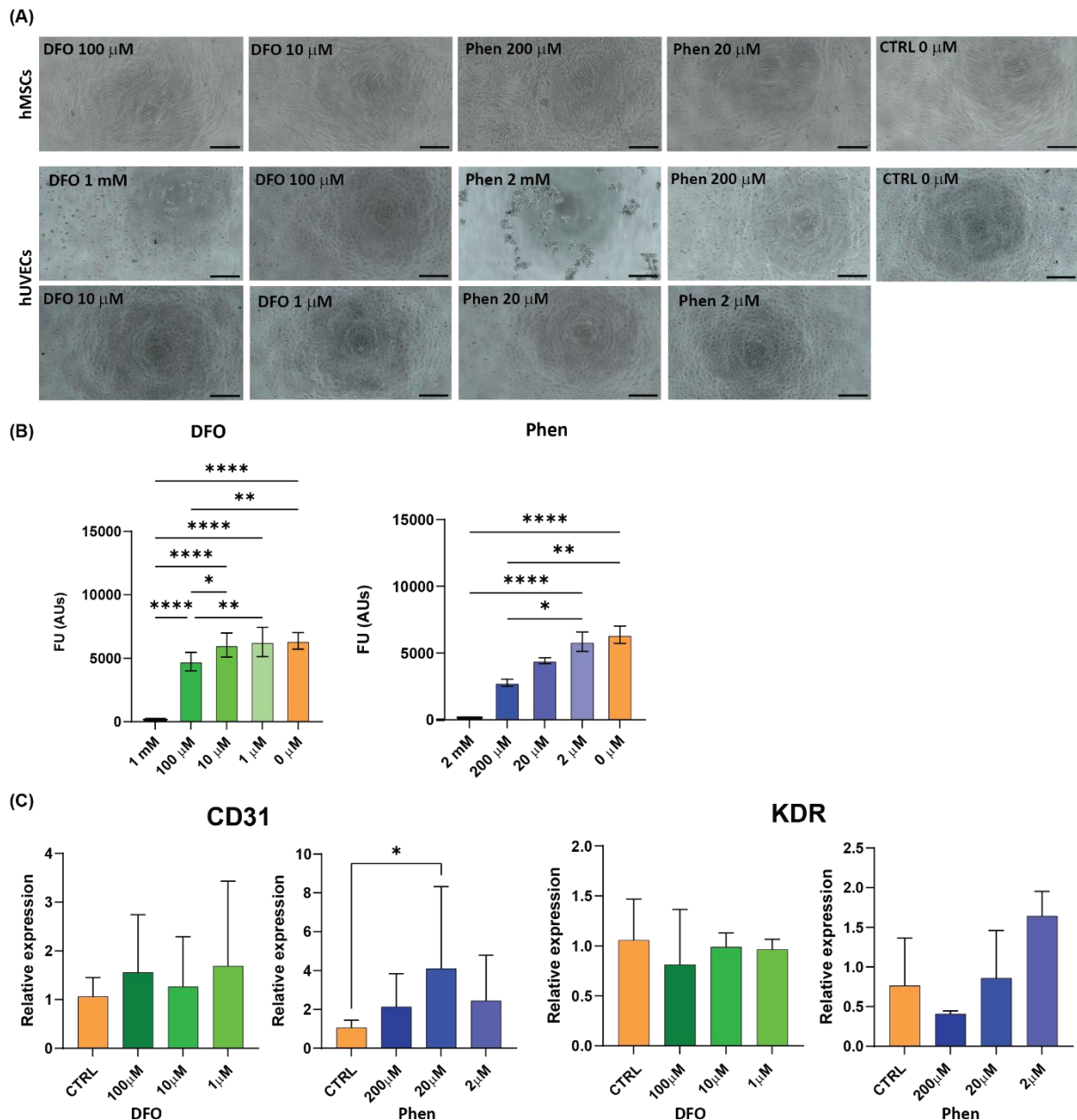


Figure S2. Evaluation of Phen and DFO effect on 2D culture of hMSCs and hUVECs. (A) Representative brightfield images of hMSCs and hUVECs exposed to different Phen and DFO concentration for 24h. Scale bar = 200 μ m. (B) Metabolic activity of hUVECs exposed for 24h to different Phen and DFO concentrations. (C) CD31 and KDR gene expression in hUVECs after 24h of incubation with different Phen and DFO concentrations. Statistical significance represented by: ****<0.0001, ***<0.001, **<0.01, *<0.05. P-value KDR_200 μ M vs KDR_2 μ M = 0.106.

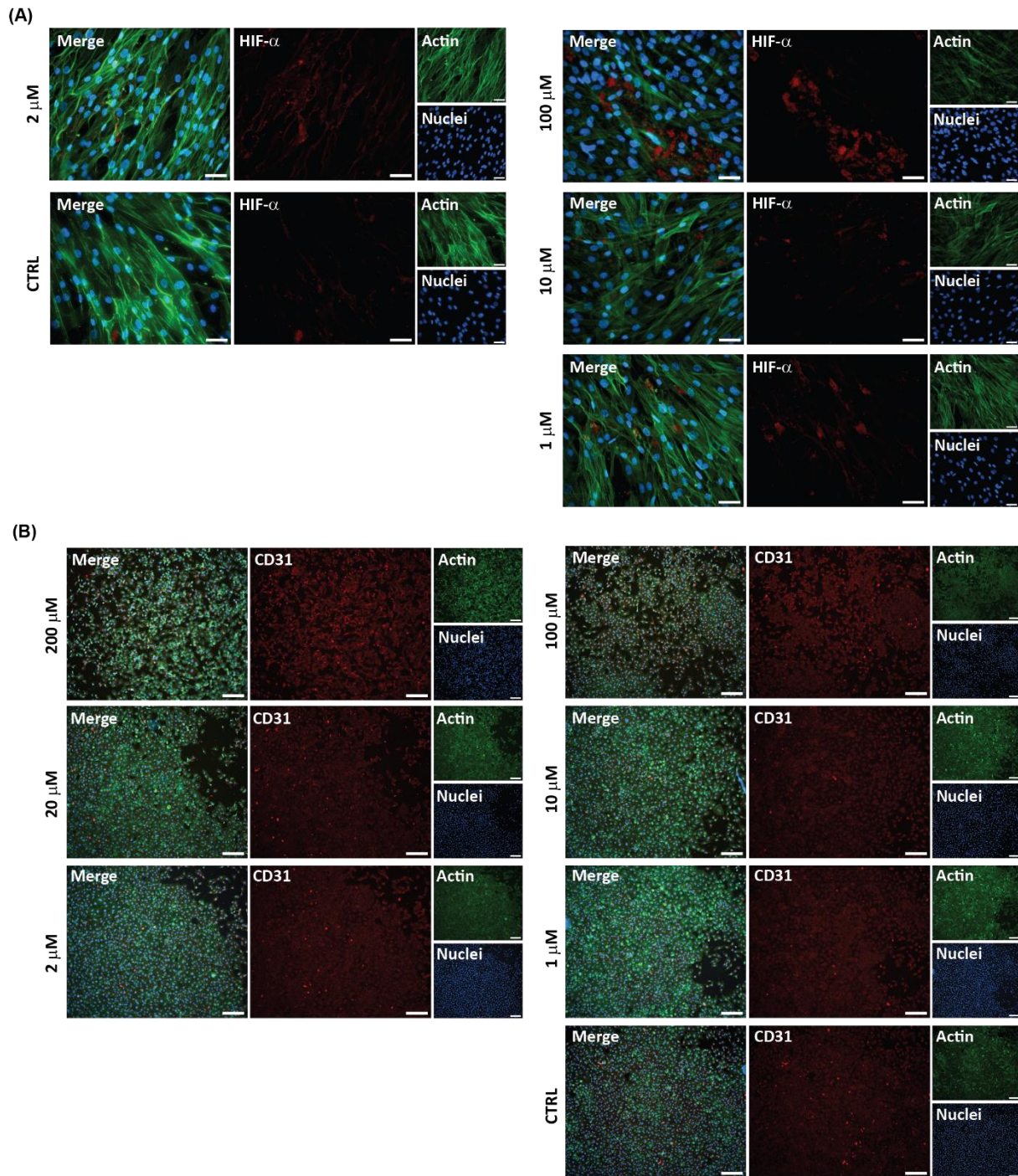


Figure S3. Evaluation of Phen and DFO effect on 2D culture of hMSCs and hUVECs. (A) Immunostaining images of hMSCs incubated for 24h with various Phen or DFO concentrations, showing HIF-alpha (red), F-actin (green) and nuclei (blue). Scale bar = 200 μ m. (B) Immunostaining images of hUVECs incubated for 24h with various Phen or DFO concentrations, showing CD31 (red), F-actin (green) and nuclei (blue). Scale bar = 200 μ m.

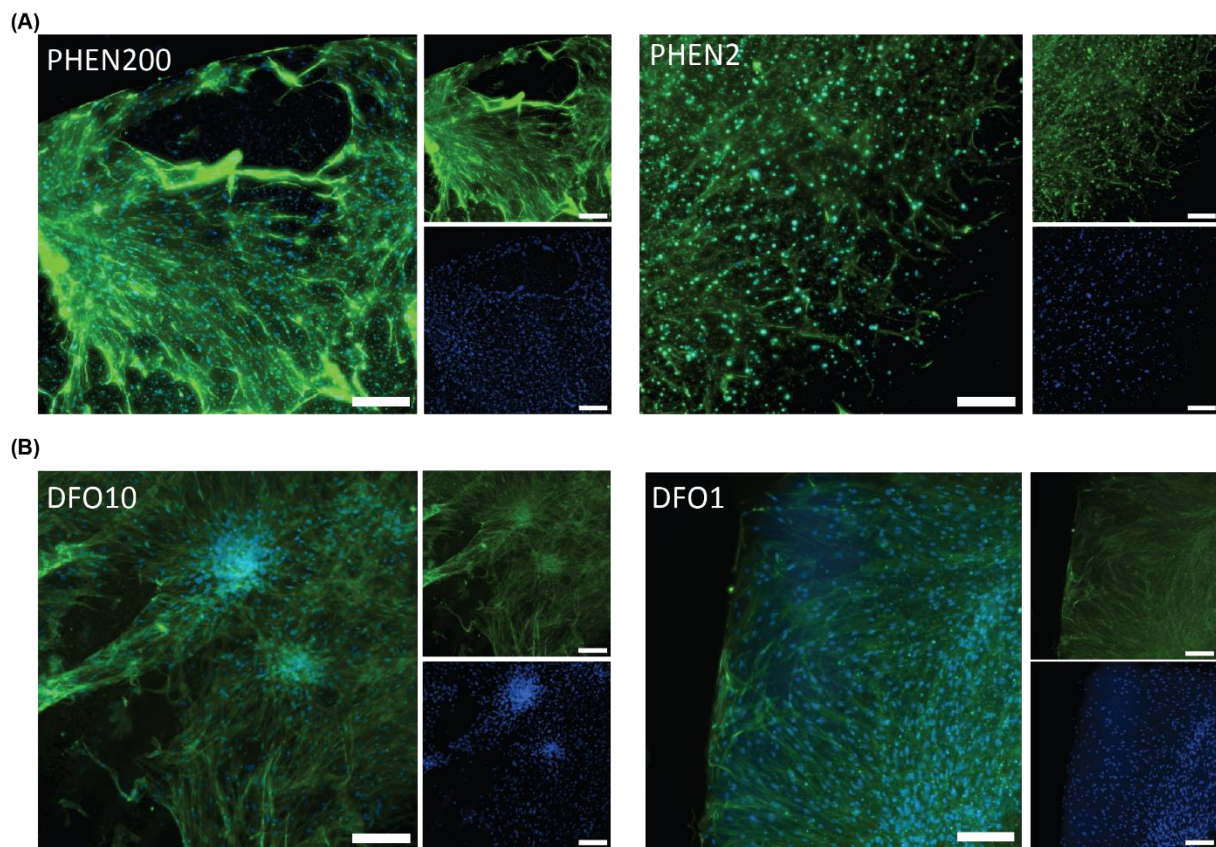


Figure S4. Evaluation of hMSCs behavior when seeded on ELR membranes loaded with different Phen and DFO concentrations. (A) Fluorescent images of ELR-Phen or (B) ELR-DFO membranes with seeded hMSCs, showing cell morphology (nuclei-blue, F-actin-green) after 7 days of culture. Scale bar = 200 μm .

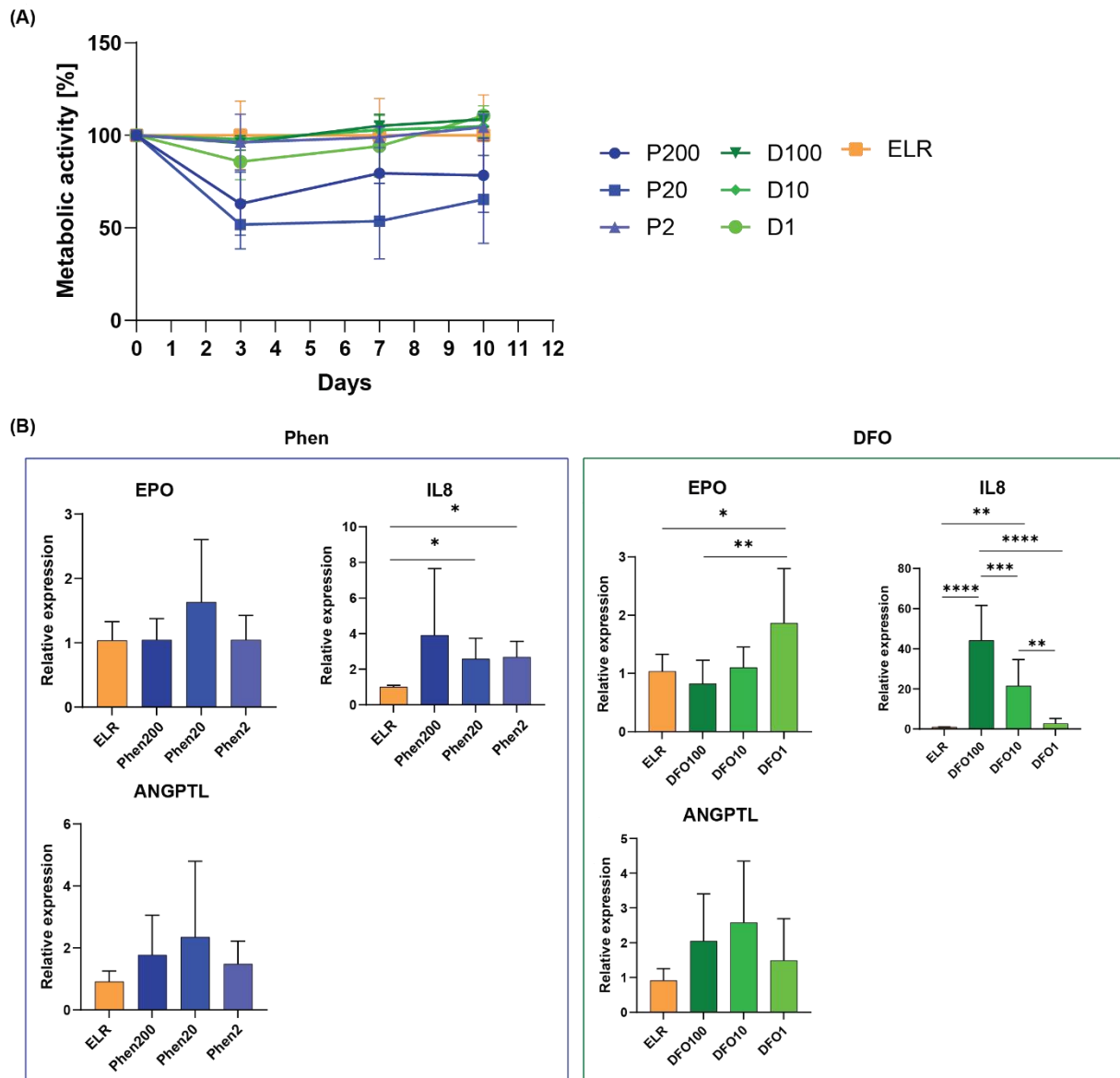


Figure S5. Evaluation of hMSCs response to the ELR-coated scaffolds at different drug concentrations. (A) Metabolic activity of hMSCs over 7 days of culture on ELR-coated scaffolds loaded with different Phen or DFO concentrations. (B) Gene expression of hMSCs of hypoxia downstream genes after 10 days of culture. ELR-coated scaffolds only were used as controls. Statistical significance represented by: ****<0.0001, ***<0.001, **<0.01, *<0.05.

CHAPTER 6

Engineering a hybrid 3D construct for bone regeneration to promote simultaneous pre-vascularization and osteogenic differentiation in vitro

Sophia Dalfino, Sveva Fagiolino, Ivo Beeren, Marco Borrone, Francesco Alviano, Carlos Mota, Gianluca Tartaglia, Claudia Dolci, Lorenzo Moroni

Submitted to Advanced Healthcare Materials (Wiley)

Abstract

Critical-sized bone defects represent a challenge in bone tissue engineering, due to insufficient vascularization that results in implant failure. Scaffold pre-vascularization is a promising strategy to create a functional microvascular network that integrates with host vasculature. In this study, we present a hybrid 3D construct comprising a hyaluronic acid-based hydrogel and a 3D printed polycaprolactone/ β -tricalcium phosphate scaffold, to support vascular network formation and osteogenic differentiation. Peptide-functionalized (i.e. RGD, YIGSR, IKVAV, QK) hydrogels were obtained via thiol-ene chemistry, using two crosslinkers (PEG-diSH or MMP-diSH). Preliminary biological experiments assessed human mesenchymal stromal cells (hMSCs), endothelial cells (hUVECs), and their co-culture, on different gel formulations. All cell conditions displayed enhanced spreading and metabolic activity on gel formulations comprising RGD; thus these (i.e. RGD only and a combination of RGD/YIGSR) were selected for further studies. Cells were then mixed with the hydrogel precursor solutions, which were injected to embed the scaffolds and crosslinked using a UV lamp. After 7 days, tubule formation was observed only in co-culture conditions, highlighting the importance of cellular crosstalk for the formation of a vascular network. Significant differences were found across the tested formulations. In the RGD-PEG constructs, hUVECs formed tubule-like structures, surrounded by hMSCs, exhibiting pericyte-like behavior, supported by the upregulation of α SMA gene. Conversely, in the RGD/YIGSR-MMP conditions, hMSCs were mostly located on the scaffold fibers, and showed the highest expression of early osteogenic markers (RUNX2 and ALP). Overall, we demonstrated that the hybrid system with tailored hydrogel chemistry can support simultaneous microvascular organization and osteogenic commitment, offering a promising platform for bone tissue engineering applications. However, further studies involving longer culture periods will aim at clarifying the complex interplay between material composition, cell crosstalk and spatial organization and their influence on the maturation and stability of the vascular network.

Keywords

Additive manufacturing, Cell-laden hyaluronic acid hydrogel, Complex geometries, Composite scaffolds, Bone tissue engineering

Introduction

Mandibular bone defects arising from mandibular resections are nowadays repaired with fibula flap grafts, the clinical golden standard treatment [1]. However, many limitations are associated with this highly invasive intervention, such as limited availability, and donor site morbidity [2]. Bone tissue engineering (BTE) offers an alternative solution to repair such defects, based on synthetic grafts (i.e. scaffolds), which can be combined with cells or bioactive factors to guide tissue regeneration. Additive manufacturing (AM) techniques are commonly used to fabricate 3D scaffolds for mandibular applications, as they allow control over scaffold design and mechanical properties, and processability of broad range of materials [3]. Despite many advances have been made in enhancing bone formation by optimizing composite materials via the addition of inorganic components such as calcium phosphates [4,5], as well as the scaffold geometry [6,7], a major limitation in clinical translation remains lack of vascularization of the 3D constructs [8]. Blood vessel infiltration from the host tissue is slow (5-17 $\mu\text{m}/\text{h}$) and reaches only a depth of a few hundreds of micrometers [9,10], whereas tissue engineered constructs span typically from a few millimeters up to a centimeter in length and/or height. Without sufficient blood perfusion, from early stages after implantation onward, these 3D constructs cannot support bone formation. Therefore, their angiogenic properties need to be improved to promote vascularization throughout the 3D constructs.

Several approaches have been proposed to enhance angiogenic processes, such as transfecting host cells by inducing an overexpression of pro-angiogenic factors [11,12], or engineering materials that can release pro-angiogenic ions or growth factors over time [13]. However, these approaches usually rely on the body response in activating and recruiting cells, which typically results in slow vascularization [14]. Moreover, the angiogenic factors are usually released in a short time window, limiting their long-term effect, which may be associated to incomplete vascularization [15]. To overcome these issues, one interesting approach is *in vitro* pre-vascularization of 3D constructs, encapsulating co-cultures of human endothelial cells (hUVECs) and stromal cells, which allows the formation of a microvascular network prior implantation that can later inosculate with the host vasculature [16,17]. This approach could offer a potentially less invasive semi-autologous alternative to the fibula flap grafts for mandibular reconstruction.

Hydrogel-based constructs are suitable candidates as 3D matrices that support such co-cultures, as they can mimic the natural extracellular matrix (ECM) environment in terms of mechanical and biological properties. Moreover, the hydrogel network can be modified with bioactive factors or peptides that can direct cell behavior [18]. Several naturally derived hydrogels such as alginate, gelatin, hyaluronic acid and collagen were reported in the context of vascularization for bone application [19,20]. Among them, hyaluronic acid (HA) represents a promising option since its natural abundance in human ECM.

Importantly, HA is degradable by stromal cells, such as human mesenchymal stromal cells (hMSCs), which through hyaluronidase secretion can remodel the matrix and form cellular networks. Moreover, HA degradation products have been shown to possess pro-angiogenic and osteo-inductive properties [21–23]. HA can also interact with the CD44 surface receptor group of hMSCs promoting their proliferation and migration [24]. From a chemical perspective, the HA backbone can be easily modified with motives (e.g. RGD) for focal adhesion formation, enhancing its adhesive properties.

Hydrogels are typically too soft to be applied in a high loading environment such as bone [25]. Embedding them in 3D polymeric scaffolds reinforces their mechanical properties of multiple magnitudes, creating suitable hybrid constructs to promote both vascularization and osteogenic differentiation. For example, PLA scaffolds embedded in a GelMA-based hydrogel were shown to simultaneously mechanically reinforce the construct and to improve human adipose-derived stem cells osteogenic differentiation [26]. *Li et al.* presented a PCL-Maleimide electrospun scaffold covalently linked to a HA-SH hydrogel to co-culture ADC spheroids and endothelial cells to induce vascular-like network formation [27]. More recently, *Neves et al.* presented different seeding approaches of endothelial and stromal cells to induce vascular network formation in hybrid constructs made of a PEOT/PBT scaffold and an RGD-modified pectin hydrogel [28].

Most of the studies in the context of pre-vascularization primarily focus on hydrogel properties to support endothelial organization in the 3D constructs but often overlook the osteogenic potential of the synthetic support structure. In a previous study from our group, we designed composite scaffolds comprising of polycaprolactone (PCL)/ β -tricalcium phosphate (TCP, 40% w/v), with a diamond shaped pore geometry, which successfully promoted osteogenesis in hMSCs [29]. In this study, we built upon this work by integrating a chemically modified HA-based hydrogel with the AM scaffold, to enable facile tunability of its biological properties. The HA possesses both maleimide and norbornene units. This dual chemistry strategy allowed independent peptide functionalization and crosslinking, thus permitting controlled modulation of biochemical and mechanical properties. This modular approach introduced high degree of tunability, overcoming the limitations of traditional systems where both mechanisms occur on a single reactive group, reducing control over matrix design [30,31]. In our system, we selectively grafted bioactive peptides via thiol-Michael addition reactions and induced hydrogel formation using different crosslinkers (polyethylene glycol- and matrix metalloproteinase-based) via thiol-ene chemistry, respectively.

The scope of this study was to fundamentally investigate the role of different hydrogel chemistries, moving beyond the commonly used RGD only modifications [32–34], and their interplay with osteogenic cues from the AM scaffold, on early endothelial organization and initial osteogenic commitment of human mesenchymal stromal cells (hMSCs). Accordingly,

we performed short-term cell culture experiments to evaluate the early phases of cellular response. First, we assessed the effect of different hydrogel formulations on spreading and metabolic activity of hMSCs, hUVECs and their co-culture. We selected hMSCs and hUVECs as a well-established cell model, combining osteogenic and pericyte-like potential of hMSCs with the widely characterized hUVECs, which are considered the gold standard in tubule formation studies. The most promising cell-laden hydrogel formulations were then used in hybrid scaffolds to assess their pre-vascularization and osteogenic capacity through fluorescent imaging and gene expression. To the best of our knowledge no studies report the use of composite scaffolds with advanced geometries in combination with such a hydrogel.

Materials and methods

Materials

All materials were purchased from Merck, unless stated otherwise.

HA modification

HA-norbornene (Nb)-maleimide (Mal) was synthesized following a two-step reaction (**FigureA**), by adapting a previously established procedure [35].

Hyaluronic acid-norbornene (HA-Nb)

In a typical reaction, sodium hyaluronate 200k (600 mg, 1.5 mmol, 1.0 equiv of carboxylic acid, Lifecore Biomedical, HA200K-5) was dissolved in 0.1 M 2-(N-morpholino) ethanesulfonic acid (MES) buffer (0.3M NaCl, pH 6.7), at 1% w/v. Then, 4-(4,6-Dimethoxytriazin-2-yl)-4-methylmorpholinium chloride (DMTMM, 830 mg, 3 mmol, 2 equiv) and 5-Methylamine-2-Norbornene (385 μ L, 3 mmol, 2 equiv) were added to the solution. After stirring overnight, the product was precipitated in cold ethanol, washed with EtOH (3x), and washed with diethyl ether (3x). The material was re-dissolved in distilled H₂O (*d*H₂O), transferred to a 3kDa molecular weight cutoff (MWCO) Snakeskin dialysis membrane, and dialysis was performed against decreasing NaCl concentration in *d*H₂O (from 50 mM to 0 mM, 3 water changes a day). The purified material was obtained as a white fluffy solid after freeze-drying and validated via ¹H NMR. Final yield = 480 mg, 80%.

Hyaluronic acid – norbornene – maleimide (HA-Nb-Mal)

Subsequently, in a typical reaction, the lyophilized HA-Nb (440 mg, 1.1 mmol, 1.0 equiv of carboxylic acid groups) was dissolved in degassed MES buffer at 1% w/v. Then, DMTMM (304 mg, 1.1 mmol, 1 equiv) and 2-maleimidoethylamine trifluoroacetate salt (283 mg, 1.1 mmol, 1 equiv) were added. The solution was shortly purged with dry N₂ gas and left

stirring overnight. The product was purified via precipitation in cold ethanol, wash in cold EtOH (3x) and diethyl ether (3x). The product was re-dissolved in MilliQ water, transferred to a 3kDa molecular weight cutoff (MWCO) Snakeskin dialysis membrane and dialyzed against decreasing NaCl concentration in MQ (from 50 mM to 0 mM, 3 water changes a day). The purified material was obtained as a white fluffy solid after freeze-drying and validated via ^1H NMR. Final yield = 313 mg, 72%.

Peptide functionalization

Different peptides were selected to be grafted on the polymer and their sequences are listed in **Table 1**. A schematic of the reaction is represented in **FigureB**. All peptides were obtained from Synpeptide Co., Ltd (Purity > 98%) and possessed a terminal cysteine terminal to enable coupling via thiol-Michael addition reaction to the polymer backbone. Before conjugation, the peptides were incubated in PBS containing a 10-fold molar excess of tris(2-carboxyethyl) phosphine (TCEP) for 1h at 37°C to remove the spontaneously formed disulfide bonds (5 mM peptide concentration). Then, 400 μL of the peptide solution(s), 1000 μL HA-Nb-Mal previously dissolved in PBS (4% w/v), and 600 μL of PBS was combined to obtain a 1:1 peptide – maleimide stoichiometry. The solution was left for 1h at RT. Excess of TECP was removed with ultrafiltration with a 3kDa MWCO (Amicon Ultra, Millipore) filters, according to manufacturer instructions.

Table 1. Peptides used to functionalize the Ha-Nb-Mal, via Thiol-Michael addition chemistry. 1.0 equivalent of peptides was added from a stock solution (5 mM), relative to Mal groups.

Peptide	Sequence	MW (mg/mmol)	Main function
RGD	CGGGRGDS	707,7	Integrin-binding domain
IKVAV	CGGGIKVAV	802,97	Laminin-derived
YIGSR	CGGGYIGSR	868,94	Laminin B1 chain
QK	CGGGKLTWQELYQLKYKGI	2185,51	VEGF mimetic peptide

HA characterization

The purified HA-Nb, HA-Nb-Mal and the HA functionalized with peptides were characterized to assess functionalization and purification process.

^1H -NMR

The samples were dissolved in phosphate buffered D_2O (pH 7.4, [phosphate = 56.9 mM, and [3-(trimethylsilyl)-1-propanesulfonic acid- d_6 sodium salt] = 0.11 mM). All ^1H -NMR spectra were recorded at 299.7 K with a Bruker Avance III HD 700 MHz spectrometer, comprising a cryocooled three-channel TCI probe. The spectra were processed and analyzed with TopSpin 4.0 software (Bruker, Germany). The degree of substitution for Nb and Mal were determined by the ratio between the integral values of the non-anomeric

backbone protons in the repeating disaccharide unit of HA and the integral value of either the vinylic Nb group (5.9-6.4 ppm) or the Mal group (6.85-6.95 ppm).

Gel permeation chromatography (GPC)

Gel permeation chromatography was used to determine the molecular weight and dispersity of the HA(-derived) polymers before and after the peptide functionalization. Samples were dissolved in a 0.1 M NaNO₃ solution (1mg/ml), the mobile phase of the system. All samples were filtered through filters with pore size 0.2 µm prior to injecting a volume of 50 µL. Measurements were performed on a Prominence-I LC-2030C3D LC (Shimadzu) system comprising an autosampler, a Shodex SB-G 6B guard (6.0 mm x 50 mm) column, followed by a Shodex SB-803/SB804 HQ (8.0 mm x 300 mm) columns, a refractive index detector, and a photodiode array detector. The flow rate was kept at 1 ml·min⁻¹ at T = 25 °C. Calibration was performed using PEG standards up to 545,000 MW (PEG calibration kit, Agilent Technologies).

HA hydrogel formation and characterization

In a typical procedure, HA-peptidex hydrogels were prepared by dissolving the polymer in PBS. Subsequently, the dissolved polymer was mixed with the crosslinker ([-SH] = 25 mM) and the photo-initiator to reach a final polymer concentration of 2% w/v. Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) was used as photoinitiator [2 mM final concentration]. Two crosslinkers were compared along the study, PEG dithiol (5k Biopharma PEG, HO003003-5K) and a custom-made cysteine terminal MMP2/9 cleavable peptide (GenScript, purity > 96%). The thiol to Nb were reacted in a 1-to-1 equivalent ratio to the Nb groups. Finally, 50 µl of polymer mix was cast in a mold and irradiated with a UV lamp (λ = 365 nm, I = 10 mW/cm², 20s). For the swelling test a silicon mold was used to form the hydrogels. For the cell culture experiments, the gels were formed directly in the wells of a black 96-well plate with transparent glass bottom.

Swelling/degradation

All HA gels formulations were tested for swelling over 7 days, by changing the crosslinker. The test was conducted in PBS at 37 °C.

Briefly, HA-peptidex was dissolved in PBS, then the crosslinker and the photoinitiator were added. 50 µl of solutions was cast in a silicon mold and irradiated with a UV light (λ = 365, I = 10 mW/cm², 20s exposure). Hydrogels were produced in triplicates per condition. Subsequently, the samples were placed in pre-weighted containers and submerged with PBS supplemented with PenStrep. At different time points, the solution was discarded and the samples weighed. The water uptake was calculated with the following formula:

$$\text{Water uptake (\%)} = \frac{W_t - W_0}{W_0} \times 100$$

Where W_0 is the initial mass of the hydrogel immediately after cross-linking and W_t is the mass of the gel at each time point.

Rheology

Photo-rheology was conducted on all HA-peptide formulations. Gel formulations were prepared according to the earlier described protocol and crosslinked in-situ with PEG/MMP-diSH (n=3). In short, the polymers were dissolved in PBS overnight at 4 °C. Then, the crosslinker and LAP were added to the solution before measuring. The measurements were performed on a DHR2 instrument (TA Instruments), using a cone-plate geometry (20 mm plate diameter, 2.002° cone angle) at 20°C, to mimic crosslinking conditions. The instrument was equipped with a UV light source at 365 nm and the intensity was set at 10 mW/cm². All samples undergone the same 4 step procedure: (1) time sweep for 5 min at 1 rad/s and 1% strain, after the first minute, UV was applied for 1 minute; (2) oscillation frequency 100-0.1 rad/s, 1% strain; (3) oscillation amplitude 1 rad/s, 0.1-1000% strain.

Hybrid construct fabrication and characterization

For the hybrid constructs, melt-extrusion scaffolds were fabricated, comprising medical grade polycaprolactone (PCL, Corbion) and β -tricalcium phosphate (TCP, Kuros Bioscience) in a ratio of 60:40, as described elsewhere [29]. A biomimetic diamond-shape pore geometry was chosen. Scaffolds of 6mm (diameter) x 2mm (height) were printed for biological studies, while 6mm x 4mm samples were used for mechanical testing.

Mechanical testing

The mechanical performance of the hybrid constructs upon compression was determined with a TA ElectroForce system (TA Instruments), with a 450N load cell, and controlled with Win7 software. HA-Nb was used to produce hybrid constructs for mechanical testing. The polymer was dissolved in PBS, mixed with PEG/MMP-diSH crosslinker and LAP and cast on the scaffolds placed in a silicon mold. The hydrogels were crosslinked with a UV lamp at 10 mW/cm². The samples were compressed at a strain rate of 0.04 mm/s up to a maximum of 50% compression. Stress values were obtained dividing the measured load against the apparent area of the constructs. The compressive modulus was calculated between approximately 5–15% strain in the linear region of the stress-strain curves. The yield stress and strain values were calculated with the 0.2% offset method. The toughness of the samples was approximated as the area under the curve in the elastic region.

Cell culture

Human mesenchymal stromal cells derived from bone-marrow (hMSCs, PromoCell, lot # 439Z037.1) were expanded at a density of $1,000 \text{ cells}\cdot\text{cm}^{-2}$ in α -minimum essential medium (α -MEM) (Gibco) supplemented with 10% v/v fetal bovine serum (FBS) and ascorbic acid (ASAP). The medium was refreshed every 2-3 days. Cells were passaged at 70-80% confluency and used at passage 5-6 for experiments.

Human umbilical vein endothelial cells (HUVECs, Lonza) were cultured at a density between $6,000$ - $10,000 \text{ cells}\cdot\text{cm}^{-2}$ in endothelial cell growth medium ready to use kit (Promocell, C-22010). The medium was refreshed every 2-3 days. Cells were used at passage 4-6 for the experiments. HUVEC-GFP (PeloBiotech, PB-CAP-0001GFP) were cultured at a density of $10,000 \text{ cells}\cdot\text{cm}^{-2}$ in endothelial growth medium (Pelobiotech, PB-CAP-02). The medium was refreshed every 2-3 days. Cells were used at passage 8-9 for the experiments. HUVEC-GFP were used for fluorescent imaging, while normal HUVECs were used for all the other biological assays.

Co-culture conditions validation

hMSCs and HUVECs were co-cultured, at a ratio of both medium and cells of 1:1, based on the literature [36,37]. The culture conditions were validated in 2D, by evaluating the expression of cell specific markers and metabolic activity. The culture conditions are summarized in **Table 2**. Cells were seeded in a treated 48-well plate at a density of $10,000 \text{ cells}\cdot\text{cm}^{-2}$.

On days 1, 3 and 7, the metabolic activity was measured with a Presto Blue™ (Invitrogen) Cell viability Reagent, according to manufacturer instructions, by incubating the samples in medium at 10% v/v for 15 min. The fluorescence was measured with a plate reader at excitation/emission wavelength 535-615 nm. After the assay, the cells were washed 3 times with PBS and fresh medium was added. Blank medium without cells was used as control.

On day 7, the samples were fixed in 4% v/v paraformaldehyde (PFA) for 10 min and washed in PBS. For the immunostaining, a 10 min permeabilization was first performed with Triton-X 0.1% v/v, followed by a blocking step with BSA 3% w/v and Tween20 0.1% v/v for 1.5 h. Then, primary antibodies and phalloidin were incubated overnight at 4°C. After washing, the secondary antibodies were incubated for 45 min and washed before adding DAPI (1:300) for 20 min. Antibodies and dilutions are reported in **Table S1**. Samples were imaged with a Slide Scanner microscope (Nikon Ti-E microscope, equipped with a Lumencor Spectra light source, an Andor Zyla 5.5 sCMOS camera, a Nikon DS-Ri2 color camera, and an MCL NANO Z200-N TI z-stage).

Table 2. Cell culture conditions validated in 2D and performed analysis.

Cell	Medium	Analysis
hMSCs	BM:EM = 100:0	Immunostaining: RUNX2, CD31/Ve-Cadherin, DAPI, Phalloidin Metabolic activity: Presto Blue
hMSCs	BM:EM = 50:50	
hUVECs	BM:EM = 0:100	
hUVECs	BM:EM = 50:50	
hMSCs:hUVECs	BM:EM = 50:50	

Cell seeding on top of the hydrogels

Cell seeding

The adhesion, viability and metabolic activity of hMSCs and HUVECs were first evaluated by seeding the cells on top of the hydrogels (**Figure S1A**). Four different conditions were tested as shown in **Table 3**. The hydrogels were prepared by casting 50 μ l of polymer solution in a well of a black 96-well plate and crosslinked with a UV lamp at 10 mW/cm² for 20 s. Then, the gels were submerged with culture medium supplemented with PenStrep 1% v/v and left to reach swelling equilibrium overnight. Subsequently, the medium was discarded, and the cells were seeded on top of the gels at a density of 1×10^5 cells·cm⁻². For each hydrogel, three cell conditions were seeded: hMSCs only, HUVECs only and their co-culture in a ratio of 1:1. HUVEC-GFP were used for imaging experiments.

Table 3. Summary of the tested conditions for the seeding on top of the hydrogels.

HA functionalization	Crosslinker	Tested cells	Cell density
HA-RGD	PEG	hMSCs	1×10^5 cells·cm ⁻²
		hUVECs	
		hMSCs:hUVECs = 50:50	
HA-RGD	MMP	hMSCs	1×10^5 cells·cm ⁻²
		hUVECs	
		hMSCs:hUVECs = 50:50	
HA-YIGSR	PEG	hMSCs	1×10^5 cells·cm ⁻²
		hUVECs	
		hMSCs:hUVECs = 50:50	
HA-YIGSR	MMP	hMSCs	1×10^5 cells·cm ⁻²
		hUVECs	
		hMSCs:hUVECs = 50:50	

Adhesion and viability

Cell adhesion and viability after 24h from the seeding were investigated with brightfield/fluorescent imaging and fixable live/dead immunostaining. Briefly, the samples were washed 2x with PSB, and the Fixable L/D solution was added and incubated in dark for 25 min, according to manufacturer instructions. Then, the solution was washed 2x with PBS and the samples were fixed with 4% v/v PFA for 20 min. The samples were washed again 3X and stored in PBS at 4°C for further staining. Subsequently, DAPI staining was

performed. The samples were permeabilized with 0.1% v/v Triton X for 10 min, washed with PBS 3x and stained with DAPI (1/300), before imaging with Slidescanner Microscope. The percentage of alive cells (Live %) was quantified in different conditions, by image analysis with the software *ImageJ*. Briefly, the number of dead and total cells was quantified by counting the dead-positive and Dapi-positive nuclei. Then, the Live% was calculated with the following formula:

$$Live\% = \frac{(Total\ nuclei - Dead\ nuclei)}{Total\ nuclei} \times 100$$

The percentage of alive cells in the RGD and YIGSR conditions were compared. After 24h, the covered area % in all culture conditions was also quantified. The brightfield images were processed on *ImageJ*, to quantify the area occupied by the cells. Then, the cell area was divided by the total area of the image and the covered percentage was calculated. The values were plotted to compare the effect on the peptide on the cell adhesion.

Metabolic activity and spreading

On day 1, 3 and 7 the metabolic activity (n=3) was measured by incubating a 10% v/v Presto Blue solution for 15 min, followed by measurements of fluorescence with plate reader. Moreover, on day 7 the samples were also fixed with 4% v/v PFA for fluorescence staining. Fixed samples were permeabilized with 0.1% v/v Triton X for 10 min and phalloidin was incubated overnight at 4°C. Then, the samples were washed with PBS and DAPI staining (1/300) was performed for 20 min. The samples were imaged with a Slidescanner Microscope to investigate cell spreading and morphology after 7 days of culture on different gel conditions. Detailed list of antibodies and dilutions is provided in **Table S1**.

Cell encapsulation in the hybrid scaffolds

Cell encapsulation in the hydrogels only

First, the morphology and metabolic activity of cells was evaluated, when encapsulated in HA-RGD or HA-RGD/YIGSR (ratio 1:1) hydrogels only, crosslinked with either PEG or MMP, as summarized in **Table 4**.

Hydrogel precursor solutions were prepared and mixed with crosslinkers, LAP and cells, at a density of $5 \cdot 10^6$ cells·ml⁻¹. Then, 50 µl of solution was cast in a black 96-well plate and crosslinked with a UV lamp at 10 mW/cm² for 20s (**Figure S1B**). The gels were submerged with medium, that was replaced every 2-3 days.

On day 1, 3 and 7, a Presto Blue™ assay was performed to evaluate the metabolic activity on the cells over time. Briefly, a 10% v/v of Presto Blue reagent in medium solution was prepared and incubated with the samples for 2h, to allow complete diffusion. Then,

100 μ l in duplicates were transferred to a black-bottom 96-well plate and the fluorescent was recorded with a plate reader. The samples were thoroughly washed with PBS and new medium was incubated. Gels without cells were used as blanks, and their fluorescent values were subtracted by the cell signals.

Moreover, on day 1, 3 and 7, cell morphology and organization were monitored with a Live Cell microscope. On day 7, the samples were fixed in 4% v/v PFA and stained with phalloidin and DAPI, as described above. Fluorescent images were taken with a Laser Confocal microscope. List of antibodies and dilutions is reported in **Table S1**.

Table 4. Summary of the tested conditions for cell encapsulation in the hydrogels only and in the hybrid scaffolds.

HA functionalization	Crosslinker	Tested cells	Cell density
HA-RGD	PEG	hMSCs	$5 \cdot 10^6$ cells·ml ⁻¹
		hUVECs	
		hMSCs:hUVECs = 50:50	
HA-RGD	MMP	hMSCs	$5 \cdot 10^6$ cells·ml ⁻¹
		hUVECs	
		hMSCs:hUVECs = 50:50	
HA-RGD/YIGSR	PEG	hMSCs	$5 \cdot 10^6$ cells·ml ⁻¹
		hUVECs	
		hMSCs:hUVECs = 50:50	
HA-RGD/YIGSR	MMP	hMSCs	$5 \cdot 10^6$ cells·ml ⁻¹
		hUVECs	
		hMSCs:hUVECs = 50:50	

Cell encapsulation in the hybrid constructs

Next, cells were encapsulated in hybrid constructs, comprising a PCL/ β -TCP scaffold and HA-RGD or HA-RGD/YIGSR (1:1 ratio) hydrogels, crosslinked with either PEG or MMP (**Table 4**). Prior to cell seeding, the scaffolds were disinfected in EtOH 70% v/v for 20 min, washed 3 times in PBS and incubated overnight in basic cell culture medium (alpha-MEM, 10% v/v fetal bovine serum, 1% v/v ascorbic acid), to favor protein adsorption. Then, the scaffolds were dried on absorbent paper and placed on the bottom of a 96-well plates. Subsequently, hydrogel precursor solutions were mixed with cells (density $5 \cdot 10^6$ cells·ml⁻¹) and 50 μ l were injected throughout the scaffolds to reach complete embedding. The hydrogels were crosslinked with a UV lamp (λ = 365 nm, I = 10 mW/cm², 30s) (**Figure S1B**). The constructs were submerged with culture medium that was replaced every 2-3 days.

On day 1,3 and 7, cells morphology was monitored with live imaging on a Live Cell microscope. On day 7, samples were fixed with 4% v/v PFA and a DAPI/phalloidin staining was performed as reported in the previous sections. A Laser Confocal microscope was used to image fluorescent samples. The antibodies and the dilutions that were used are reported in **Table S1**. Fluorescent images of the co-culture conditions were processed with ImageJ

software for background removal and subsequently analyzed with the software AngioTool (Java) to quantify vessel formation parameters (i.e. total tubule length, vessel area, and number of junctions). Confocal images from both the top and the cross section of the samples were analyzed. An average of the results from the two views was also calculated.

On day 7, qPCR was performed to evaluate gene expression in the hybrid scaffolds. Hydrogel only samples were used as reference. Briefly, samples were washed with PBS, minced, collected in 1 mL TRIzol (Invitrogen), and stored at -80°C. RNA was extracted with chloroform method, followed by overnight precipitation in isopropanol. RNA concentrations were measured using a BioDrop uLITE instrument (Scientific Laboratory Supplies). cDNA was synthesized using an iScript cDNA Synthesis Kit (Bio-Rad), according to manufacturer's instructions. Finally, qPCR was performed in 10 μ L volume, adding 4 ng of cDNA to the iQ SYBER green Supermix (Bio-Rad) and primer solution. The list of primers is reported in **Table S2**. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative gene expression.

Statistical analysis

All data were reported as means $\pm$ standard deviation. Statistical analysis was performed using GraphPad Prism. Normality of the data was first assessed. Statistical significance was determined by One-way analysis of variance with Tukey's honest significance test multiple comparison tests. If the data distribution was not normal, a Kruskal-Wallis non-parametric test was performed. (****) $p < 0.0001$, (***) $p < 0.001$, (**) $p < 0.01$, (*) $p < 0.05$.

Results & Discussion

Hyaluronic acid-Norbornene-Maleimide synthesis and characterization

HA-Nb-Mal was synthesized via a two-step reaction pathway using DMTMM as activator agent of the carboxylic acid group (**Figure 1A**). The Nb served as addressable unit for PEG-diSH or MMP-diSH to create a hydrogel network via thiol-ene chemistry, whereas the Mal groups were used to graft thiolated peptides via Michael addition reactions (**Figure 1B**).

The NMR spectrum of the HA-Nb-Mal showed the appearance of the characteristic peak of the double bond of the Nb (5.9-6.4 ppm) and the Mal peak (6.9 ppm). Evaluation of the integral ratio of the peaks compared to the HA backbone revealed a degree of substitution of norbornene of $8\pm1\%$, and maleimide $3\pm1\%$ (**Figure 1C**). Before hydrogel formation, peptides (i.e. RGD, IKVAV, YIGSR, and/or QK) were grafted on the HA-Nb-Mal. The complete disappearance of the Mal peak at 6.9 ppm (**Figure S2A**) as well as a small observed increase in molecular weight compared to pristine HA-Nb-Mal during GPC analysis (**Figure S2B**) suggest successful conjugation of the peptides.

Notably, when the peptides were not pre-grafted before hydrogel formation (see also section 3.2), crosslinking immediately occurred spontaneously. We believe the thiol groups of the crosslinker spontaneously reacted with the Mal groups, prior to UV illumination. The absence of this observation in the pre-grafted system further indicates successful peptide anchoring to the HA backbone.

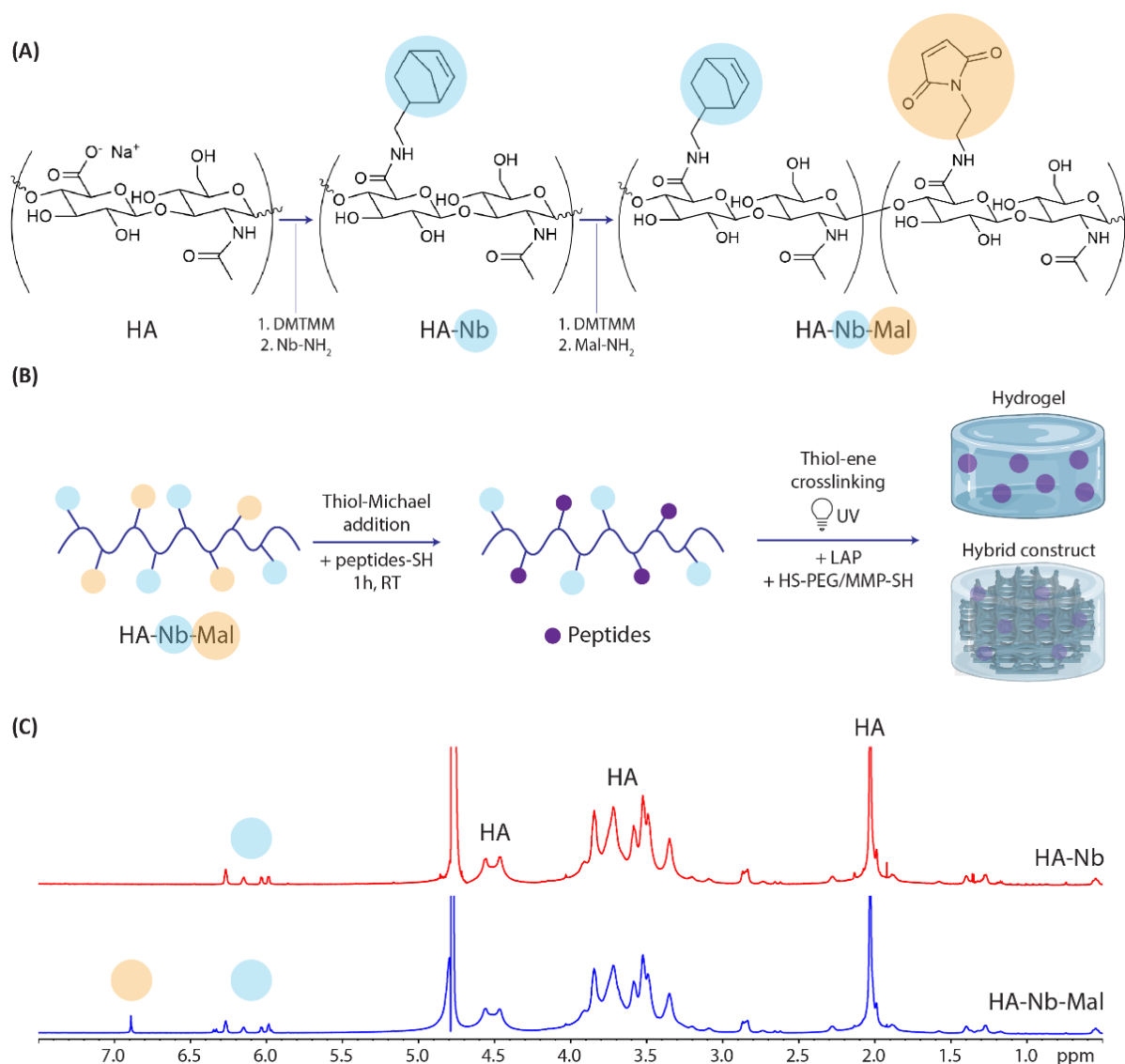


Figure 1. Schematics of reaction steps to obtain the final hydrogels. (A) HA two-steps functionalization where Nb is reacted first, followed by Mal. (B) Covalent grafting of the different peptides to the HA backbone, via Thiol-Michael addition reaction with Mal groups; thiol-ene reaction with UV to crosslink Nb groups and obtain the final functionalized hydrogel. (C) ¹H-NMR spectra of HA-Nb and HA-Nb-Mal showing the presence of Nb and Mal groups, indicating successful substitution.

Hydrogel characterization

After peptide conjugation to the maleimide unit, hydrogel formation was induced via UV using PEG-diSH or MMP-diSH as crosslinker. In preliminary tests, 1% and 2% w/v HA hydrogels were prepared using a 1:1 crosslinker (i.e. the thiol group of the PEG) versus Nb groups (**Figure S3A**). The crosslinking kinetics and viscoelastic properties of the

hydrogels were determined by photo-rheology. Compared to the 2% w/v hydrogel, the 1% w/v hydrogel was softer ($G' = 309.5 \pm 123.1$ Pa vs 1699.0 ± 315.9 Pa), did not maintain a good shape (i.e. was difficult to handle), and displayed fast degradation (< 7 days) when immersed in PBS. Thus, 2% w/v hydrogel formulation was used in the remainder of the study.

Next, the properties of HA functionalized with different peptides (i.e. HA-RGD, HA-IKVAV, HA-YIGSR, HA-QK) hydrogels and crosslinked with two different crosslinkers (i.e. PEG-diSH and MMP-diSH) were compared. Prior to applying UV, all gels remained a liquid except the HA-QK formulation that immediately gelled as indicated by the value of G' being one order of magnitude above G'' (**Figure 2A-B**). We attributed this to the significantly larger size of the peptide, resulting in a higher entanglement of the network, or electrostatic interactions resulting from the presence of charged amino acids. In addition, we observed insoluble filaments in the hydrogels after crosslinking. Regardless, uncontrolled spontaneous gelation prevented adequate handling of the formulations with QK, and these were excluded from further biological studies.

When UV irradiation was applied to all formulations, G' values rapidly increased, reaching a plateau where $G' > G''$ in less than 20 seconds (**Figure 2A-B**). This observation implies that the coupled peptides did not interfere with the thiol-ene crosslinking reaction. The stiffness of the hydrogels was extrapolated from the plateau (**Figure 2C**). The MMP-crosslinked groups displayed significantly lower G' values compared to their respective PEG-crosslinked group (e.g. HA-RGD-PEG $G' = 1699.0 \pm 315.9$ Pa vs HA-RGD-MMP $G' = 321.8 \pm 292.5$ Pa). Since the MMP-diSH is much shorter than the PEG-diSH (1041.25 g/mol vs 5000 g/mol), we hypothesized that intramolecular reactions occurred more in the MMP conditions, leading to less active crosslinkers that contribute to the stiffness of the network. When comparing the formulations comprising the same crosslinker, no significant differences were detected and the stiffness ranged from 1699 ± 315.9 to 4440 ± 527.8 Pa for the PEG group and from 321.8 ± 292.5 to 1423 ± 1366 Pa for the MMP group. Overall, all the values were in the range $10^2 - 10^3$ Pa, comparable with most soft tissues in the body [38–40].

The frequency sweep results showed that all formulations displayed frequency-independent moduli over the measured range (1-100 rad/s) (**Figure S3B-C**). In addition, the strain sweep showed typical elastic breaking behavior, with all hydrogel networks breaking around 200% (**Figure S3D-E**). This elastic behavior is typically observed in covalently crosslinked networks.

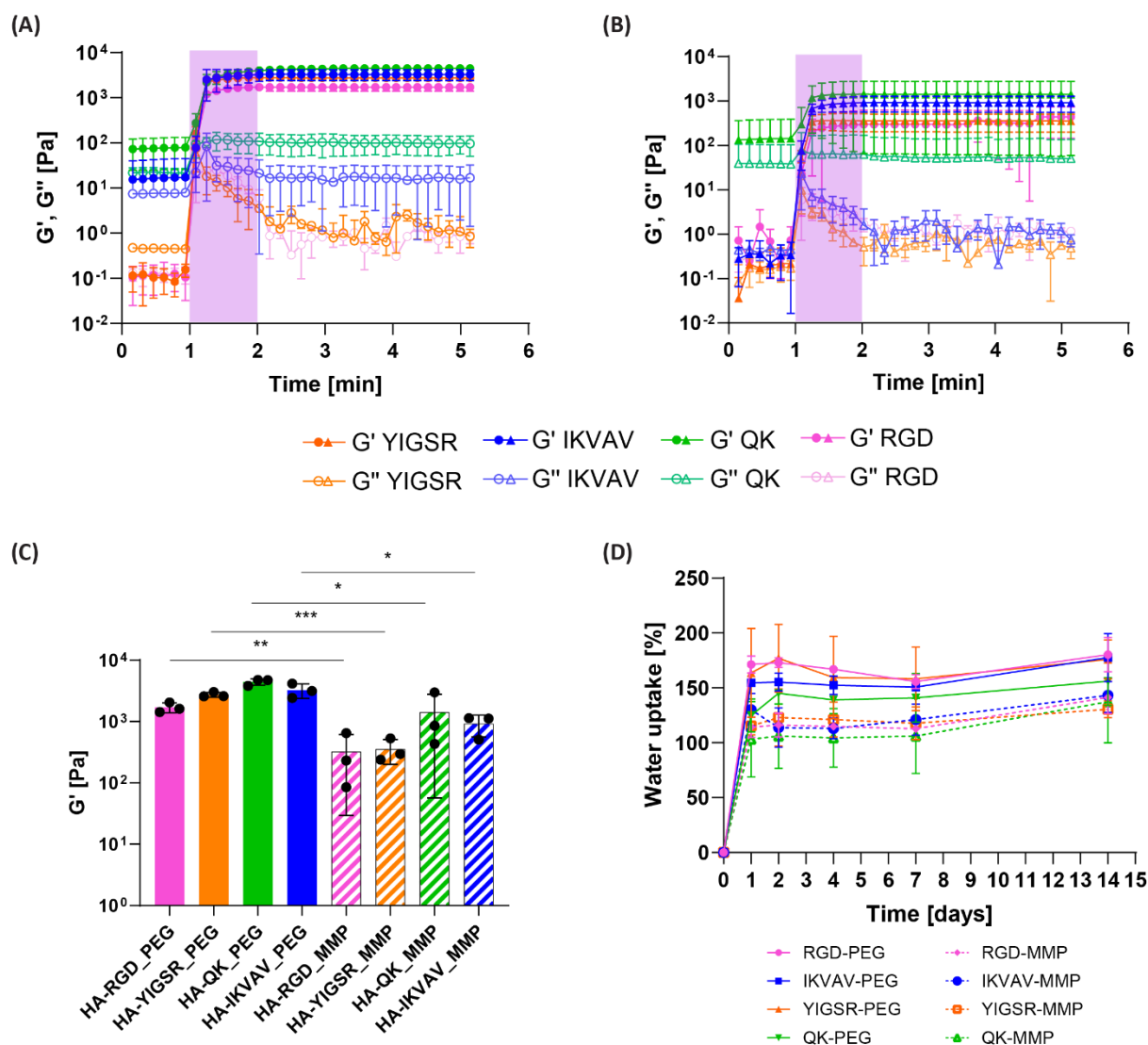


Figure 2. Characterization of the hydrogels. (A) Time sweep step of HA-peptide-PEG and (B) HA-peptide-MMP, showing storage and loss moduli, and the time window where the UV light was applied. (C) Storage moduli values of different hydrogel formulations showing the effect of peptide and crosslinker variations. (D) Water uptake percentage of different hydrogel formulations, varying the peptide and the crosslinker. Statistical significance represented by: *** <0.001 , ** <0.01 , * <0.05 .

The swelling behavior of different hydrogel formulations was tested in physiological conditions by incubating them in PBS at 37°C and refreshing the PBS every 2-3 days to simulate cell culture. All gels swelled up to around 150-200% within 24h from incubation (**Figure 2D**). While no effect was reported by varying the grafted peptide, a lower swelling was observed for the MMP group compared to the PEG one (average of $158.9 \pm 12.73\%$ versus $119.3 \pm 5.51\%$) (**Figure S3F**). The shorter length of the MMP crosslinker might result in smaller mesh size of the network, allowing lower water uptake [41]. This result aligns with what observed for the rheological behavior.

Hybrid construct characterization

Hybrid constructs comprising of the PCL/ β -TCP scaffold and a HA-peptide_x-crosslinker_y were fabricated via the injection of a liquid formulation throughout the AM construct, followed by UV crosslinking for 30 sec to ensure complete crosslinking. The injection volume was optimized to 50 μ L to ensure complete filling of the pores through all layers of the scaffold, as shown by the stereomicroscope images of both the top view (**Figure 3A**, **Figure S4A**) and the cross-section (**Figure 3B**).

Since no major differences in elastic properties of hydrogels were observed during rheology among the different peptide groups, we subjected only HA-Nb gels formulation (PEG or MMP) to a compression test. The mechanical properties of the hybrid constructs were compared to the empty scaffolds. The stress-strain curves showed a similar behavior for all conditions, with the scaffold being dominant in the mechanical behavior. This observation is well-reported in the literature, where hydrogels are reinforced to enhance their overall mechanical behavior of several magnitudes [42,43]. All curves presented an initial elastic response, followed by a plastic deformation during the plateau phase, and finally a second modulus increase due to pore closure (**Figure 3C**). The extended toe region of the hybrid construct is likely a result of the slight overfilling of the hydrogels. The following compressive moduli were determined for the constructs: $E_{\text{empty scaffold}} = 31.1 \pm 3.1$ MPa, $E_{\text{Hybrid-PEG}} = 17.7 \pm 3.5$ MPa, $E_{\text{Hybrid-MMP}} = 25.3 \pm 1.6$ MPa (**Figure 3D**). Although they are within the same regime, the addition of the hydrogel slightly reduced the compressive moduli of the construct. This observation could be explained by the dissipation of load from the scaffold to the soft gel. Also, the hydrogel may have introduced a dampening effect in the hybrid constructs, further indicated by a shift of the curves towards higher yield strain values (**Figure 3E**). Toughness and yield stress did not display significant differences (**Figure S4B-C**).

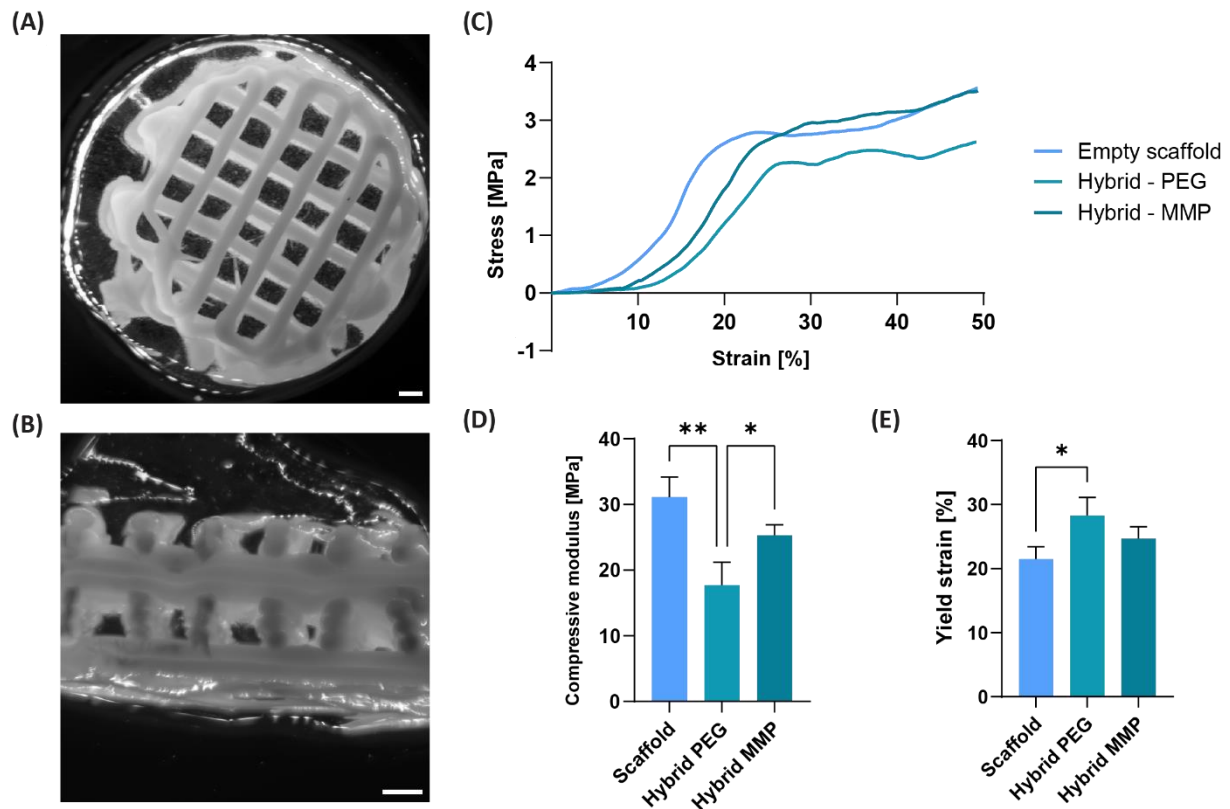


Figure 3. Characterization of hybrid constructs. Stereomicroscope image of the hybrid constructs, comprising the scaffold and the HA hydrogel, (A) from the top and (B) from the bottom. Scale bar = 500 μm . (C) Representative stress-strain curves of the scaffold only, and hybrid scaffolds crosslinked with either PEG or MMP. (D) Compressive moduli [MPa] and (E) Yield strain [%] values comparing the different conditions. Statistical significance represented by: **<0.01, *<0.05.

Cell co-culture validation

hMSCs and hUVECs have often been co-cultured to promote the formation of mature and vascular network. These cells interact through paracrine signaling of growth factors, such as VEGF and IGF, and through gap junctions, leading to the spontaneous formation of vessels [44]. Based on previous literature studies, we selected a 1:1 cell and culture medium ratio for the co-culture of hMSCs and hUVECs [44–46]. These parameters were first verified in 2D by seeding hMSCs alone, hUVECs alone, or both cells in a 1:1 cell ratio. Subsequently, their characteristics markers, i.e. RUNX2 and CD31/VE-Cad, were evaluated through immunostaining and the metabolic activity was measured (**Figure S5**).

hMSCs and hUVECs expressed their specific markers, both in monoculture and co-culture (**Figure S5A-E**). All the culture conditions displayed an increasing metabolic activity over time; hMSCs appeared more metabolically active at all timepoints than hUVECs (**Figure S5F**). In conclusion, the selected cell and medium ratio (1:1) were shown to permit cell proliferation and expression of specific markers. These culture conditions were used for all further biological characterization.

Cell adhesion and metabolic activity on top of the gels

An initial biological study was conducted to evaluate the ability of HA hydrogels to support adhesion, proliferation, and metabolic activity of hMSCs, HUVECs, and a co-culture thereof. RGD and YIGSR hydrogels were selected for this experiment, crosslinked with either PEG or MMP, as summarized in **Table 3**. RGD is known for its adhesive properties for cells, while YIGSR – a laminin-derived peptide – has been shown to promote tubule formation in HUVECs [47,48]. IKVAV, despite also being a laminin-derived peptide, showed less stable tubule networks and lower total tubule length both *in vitro* and *in vivo*, and was therefore excluded from further studies [49]. Similarly, QK, a VEGF-mimetic peptide, promoted lower tubule formation when HUVECs were seeded on Matrigel, compared to YIGSR and RGD hydrogels [50]. This, in combination with the practical handling problems reported in the rheological section, led to the exclusion of this peptide from further biological studies.

Cells were seeded on top of the different hydrogel formulations. After 1 day, cells on the RGD condition displayed good adhesion and adopted an elongated morphology for both the co-culture (**Figure 4A**) and the mono-cultures (**Figure S6A**). The covered area % of cells in different conditions showed greater coverage in the RGD gels, both PEG (RGD 12-24% vs YIGSR 5-9%) (**Figure 4B**) and MMP (RGD 18-21% vs YIGSR 6-10%) (**Figure 4C**). This data suggests that cells spread less on the gels comprising YIGSR.

In addition, the quantification of the Live cell % displayed a significantly greater number of alive cells in the RGD gels compared to the YIGSR conditions ($74.22 \pm 3.80\%$ vs $52.83 \pm 2.54\%$) (**Figure 4D**). This is likely associated with the higher adhesive properties of the gel comprising RGD and not due to toxicity of the gel itself. In YIGSR conditions, the spheroid-like structures of the co-culture comprised a necrotic core. When only HUVECs were seeded on the gels with YIGSR, no cells were left after the staining procedure. These results indicate that the HUVECs do not bind to the YIGSR moiety, preferring RGD instead (**Figure S6B**). No differences were appreciated between the PEG and MMP crosslinkers.

After 7 days, the cells displayed increasing metabolic activity, particularly for the RGD gels, and with around 40-fold higher values for the hMSCs compared to HUVECs in the RGD-PEG condition (**Figure 4E-G**). The higher amount of cells in the microscopy images revealed that the RGD gels supported proliferation. The cells on YIGSR gels maintained their spheroid-like shape, further confirming the preference for RGD (**Figure 4H, Figure S6C**).

The results of this first biological study indicated that the compositions comprising RGD peptides supported better cell adhesion, confirming the central role of the RGD complex in the integrin-mediated cell attachment [51]. On the contrary, the YIGSR hydrogels resulted in lower spreading and cells displaying spheroid-like morphology. Moreover, for the same cell condition, no significant differences were observed when comparing the two

crosslinkers (e.g. RGD-PEG-MSC vs RGD-MMP-MSC) (Figure S7). Thus, RGD-PEG/MMP conditions were moved to further studies, while the YIGSR hydrogels were excluded. Additionally, a second hydrogel formulation was introduced, HA-RGD/YIGSR crosslinked with either PEG or MMP, comprising a combination of the two peptides in a 1:1 ratio. Here, the synergistic effect between the adhesive properties of RGD and the angiogenic properties of YIGSR was investigated [49].

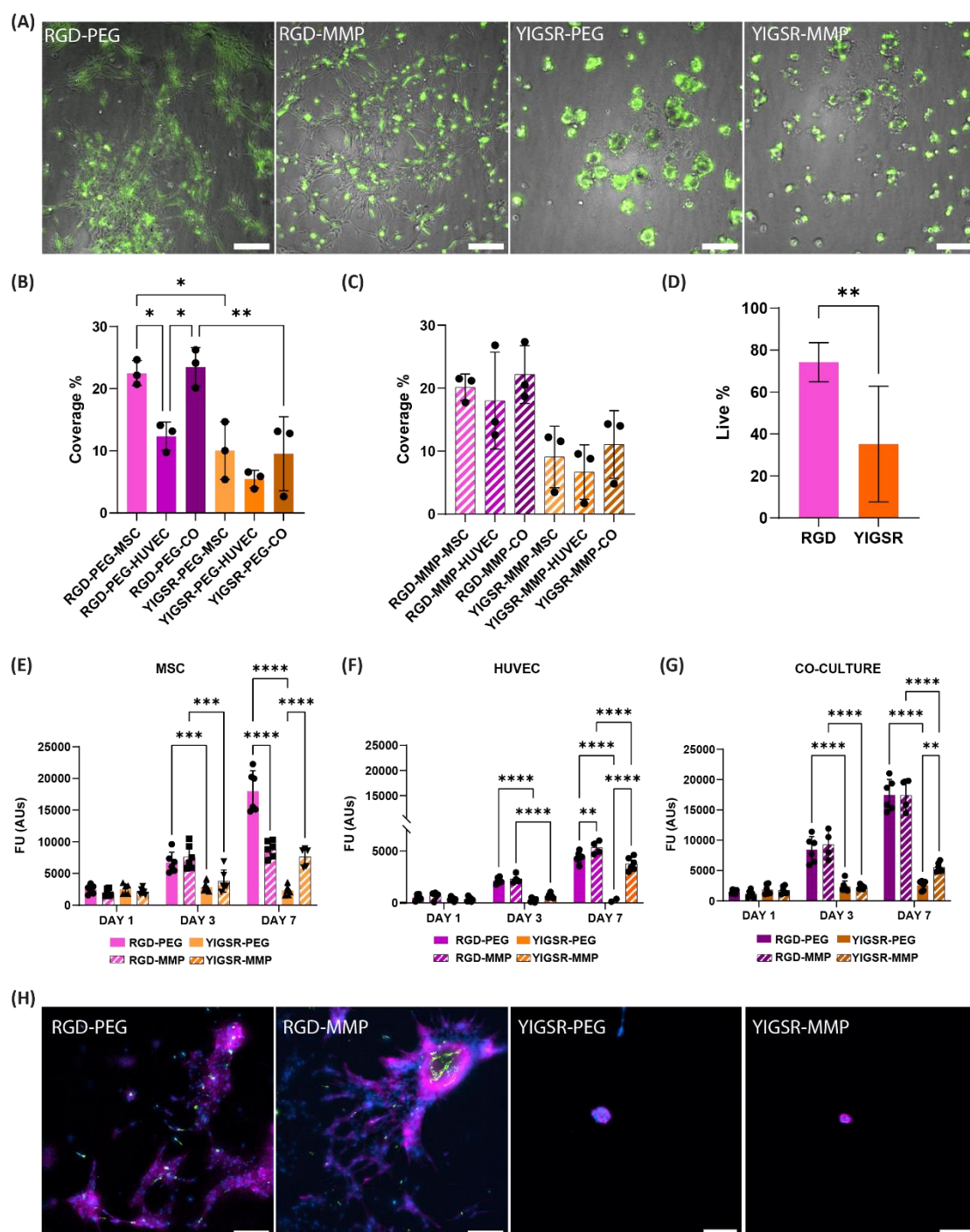


Figure 4. Assessment of cell response when seeded on top of HA-RGD-PEG/MMP or HA-YIGSR-PEG/MMP hydrogels. (A) Qualitative evaluation of the morphology of the co-culture

(hMSCs=brightfield, hUVEC-GFP=green) after 24h from the seeding on top of the gels, in different conditions. Scale bar 200 μ m. Coverage% of the cells seeded on top of the gels in (B) PEG and (C) MMP condition. (D) Quantification of the live cell% from the DAPI/dead staining, comparing RGD vs YIGSR group, to see the effect of the peptide. Metabolic activity over 7 days of (E) hMSCs, (F) hUVECs and (G) their co-culture, when seeded on different gel conditions. (H) Immunostaining of the co-culture after 7 days on different gel conditions, showing spheroid-like structures on the YIGSR conditions. DAPI (blue), Phalloidin (magenta), hUVEC-GFP (green). Scale bar 200 μ m. Statistical significance represented by: ****<0.0001, ***<0.001, **<0.01, *<0.05.

Cell encapsulation in the hydrogels

After a preliminary cell seeding on top of the gels, we encapsulated the cells within the selected hydrogel formulation (i.e. HA-RGD and HA-RGD/YIGSR, crosslinked with either PEG or MMP) to evaluate their behavior in 3D. Three different cell configurations (**Table 4**) were encapsulated by mixing the cells with the hydrogel precursor solutions, followed by casting and crosslinking with a UV lamp. Cell morphology was monitored using GFP-positive hUVECs and metabolic activity was measured over 7 days. All hydrogel formulations enhanced the metabolic activity of the cells over time. For all cell types, RGD hydrogels promoted significantly higher metabolic activity than RGD/YIGSR ones. While comparing the crosslinkers, MMP performed better than PEG (**Figure 5A, Figure S8A-B**).

On day 7, cells adopted an elongated morphology in all hydrogel conditions, suggesting attachment to the hydrogels (**Figure 5B**). When comparing the behavior of hUVECs(-GFP) versus hMSCs in the co-culture conditions, differences were observed: hUVECs remained isolated, whereas hMSCs formed endothelial-like networks throughout the gels.

Similar behavior was reported before, where hMSCs were shown to undergo mesenchymal-to-endothelial transition when exposed to endothelial cell culture medium in an ECM-like environment [52–54]. For instance, hMSCs encapsulated in a Dextran-HA hydrogel loaded with VEGF or in PEG-based hydrogel showed upregulation of CD31 and KDR after 21 days of culture, and significant matrix remodeling capacity. In the same studies, hUVECs failed to sprout in the hydrogel and presented reduce metabolic activity [55,56]. More recently, Perin et al. reported that hMSCs encapsulated in alginate-based hydrogels also formed extensive endothelial-like network, while hUVECs remained isolated in the hydrogel [30]. Indeed, when we performed gene expression analysis of cells encapsulated in different hydrogel combinations, upregulation of CD31 and KDR was observed in all cell conditions (**Figure 5C, Figure S8C**).

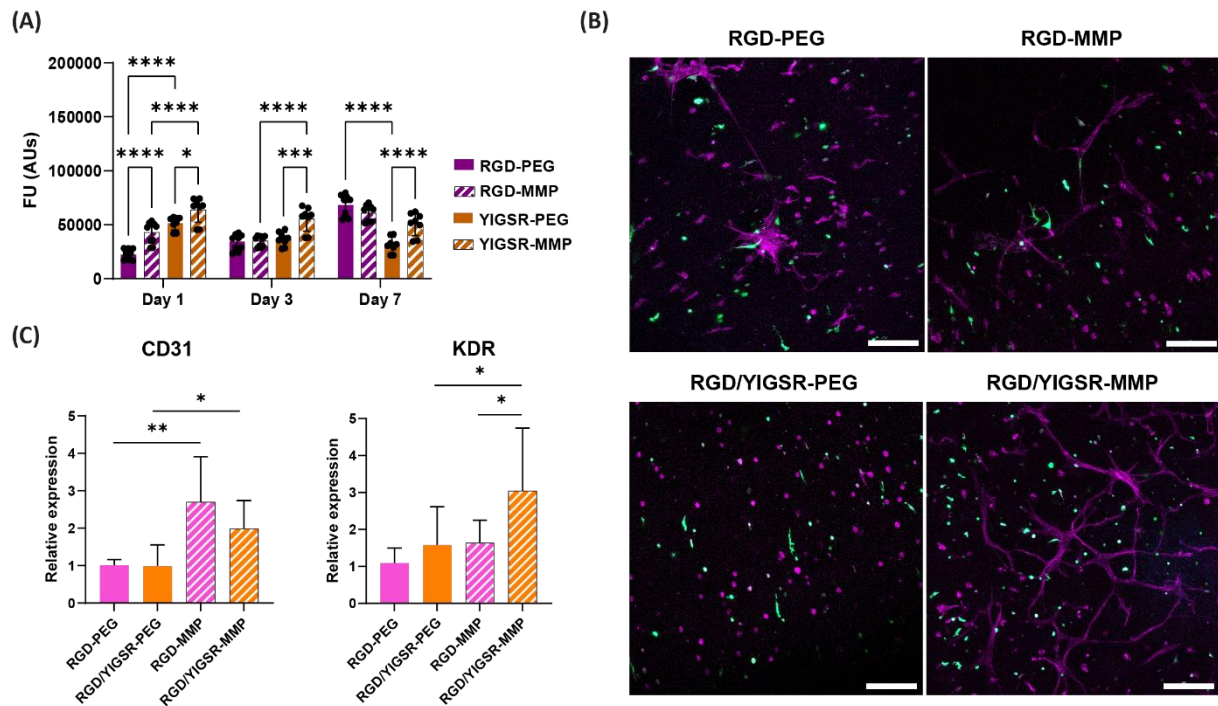


Figure 5. Assessment of the cell response when encapsulated in HA-RGD-PEG or MMP or HA-RGD/YIGSR-PEG or MMP hydrogels. (A) Metabolic activity over 7 days of hMSCs and hUVECs co-culture. (B) Immunostaining of the co-culture conditions after 7 days of culture in different gel conditions. DAPI (blue), Phalloidin (magenta), hUVEC-GFP (green). Scale bar 200 μ m. (C) Gene expression of CD31 and KDR genes in the hydrogels only for the co-culture condition. Statistical significance represented by: ****<0.0001, ***<0.001, **<0.01, *<0.05.

Cell encapsulation in the hybrid constructs

Vascularization

Next, the hydrogel precursors were mixed with cells, and dispersed throughout the scaffold, according to the steps earlier described. After 7 days, vessel formation in the hybrid constructs was evaluated via fluorescence microscopy. To enable qualitative visualization of endothelial and stromal cells, hUVEC-GFP cells were used in the experiment, as previously reported in similar hydrogel-based culture models [57]. Constructs with only hMSCs or only (GFP-)hUVECs did not display any vessel formation. hMSCs appeared to migrate towards the printed fibers while degrading the hydrogels, whereas hUVECs remained isolated in the hydrogel maintaining a rounded morphology. The failure of endothelial cell monocultures to assemble into capillary-like structures reinforces the importance of cellular crosstalk with stromal cells [58]. The combination of paracrine signaling and cell-to-cell interaction leads to recruitment of stromal cells, which are involved in matrix remodeling and stabilization of the newly formed vascular network [10].

Further supporting the hypothesis, in our study the hybrid constructs with all gel formulation comprising a co-culture induced endothelial tubule-like cell organization. Notably, from both a qualitative and quantitative assessment of fluorescent images, the

RGD-PEG conditions supported the development of significantly longer capillary-like structures throughout the scaffold thickness (**Figure 6A, Figure S10**). We observed that hMSCs were located both in proximity of the newly formed endothelial structure, supporting the early formation of tubules, and on scaffold fibers. In addition, RGD-PEG scaffolds induced significant upregulation of α SMA gene in the co-culture condition compared to the other scaffolds (**Figure 6B**). α SMA gene expression was used as a preliminary indication of pericyte-like behavior of hMSCs during the early phases of co-culture organization. This phenotype enables hMSCs to support and stabilize vascular structures, by organizing around them [59]. This was shown to keep the network stable up to 130 days *in vivo* [60]. Similar results were reported when a co-culture of hUVECs and hMSCs was seeded on poly(LLA-co-DXO) scaffolds, where α SMA positive hMSCs were identified in proximity to the newly formed tubules [61]. Hence, the upregulation of α SMA gene together with qualitative assessment of cellular organization in the RGD-PEG hybrid scaffolds was considered an initial indication of commitment of hMSCs towards pericyte-like behavior and support the observation of early vessel formation.

Zooming in on the RGD/YIGSR-hybrid constructs, the capillary-like structures appeared shorter and less developed than in the respective RGD scaffolds (**Figure 6C, Figure S10**). Similarly, the hMSCs appeared less spread and only limited contact with endothelial cells was observed. When comparing the two crosslinking methods, significantly lower tubule length, vessel area and number of junctions were recorded, only for RGD-MMP samples, which could be attributed to differences in hydrogel degradability that might have resulted in a less stable matrix for cellular anchorage.

In this study, we focused on capturing the initial phases of cellular spatial organization within the hybrid system over a 7-day culture period. For this reason, α SMA gene expression was used as an early indicator of pericyte-like behavior of hMSCs, allowing us to evaluate the effect of the different hydrogel chemistries on the expression of pericyte-related markers. The authors acknowledge that the expression of additional markers should be evaluated to further confirm a fully developed pericytic phenotype, including the presence of contractile markers such as PDGF- β or NG2, as well as the formation of a lumen and CD31/ α SMA co-localization [28,62,63]. However, these markers are typically observed at later stages of vascular network maturation, which were not reached within the scope of this study. Future studies could therefore aim to assess longer culture periods to evaluate full network functionality.

Osteogenic capacity

Next to promoting vascularization, hybrid scaffolds were designed to possess osteogenic properties, to simultaneously support bone formation. To this end, we have included in the constructs an AM scaffold comprising PCL/ β -TCP, which has been shown to

promote osteogenesis in earlier studies [29]. Gene expression of early osteogenic markers (i.e. RUNX2 and ALP) was assessed. Both genes were upregulated in the hybrid constructs when compared to the hydrogels alone, supporting the hypothesis that the scaffold embedded in the gel matrix, not only contributes to mechanical reinforcement, but also guides cells (i.e. hMSCs) towards an osteogenic lineage (**Figure S9A**).

When comparing the gene expression across hybrid scaffold conditions, we observed a 4-fold upregulation of RUNX2 and 6-fold upregulation of ALP in the RGD/YIGSR-MMP hybrid scaffolds (**Figure 6D**). The cells in the respective RGD/YIGSR-PEG only presented around 2-fold upregulation of osteogenic genes, suggesting that the strongest osteogenic properties might arise from the combination of the scaffold and the degradable MMP crosslinker in the hydrogel. These observations highlight that the hydrogel composition is relevant and can be tailored to guide vascularization and osteogenic processes.

Our findings align with other literature studies. For instance, Sousa et al. showed that PCL-nHA scaffolds embedded in alginate/collagen-based hydrogels improved mineralization of hDPSCs spheroids *in vitro* and significant tubule formation *in vivo* when implanted in a subcutaneous rat model [64]. Dubey et al. fabricated melt-electrowritten PCL scaffolds reinforced by a GelMA-based hydrogel and demonstrated that the direct co-culture of hMSCs and hUVECs significantly improved mineralization and upregulated the expression of several osteogenic genes [65]. We hypothesize that the presence of stiffer AM scaffolds with defined fiber orientation provides physical cues that in combination with cellular crosstalk and a defined hydrogel chemistry promote osteogenic differentiation.

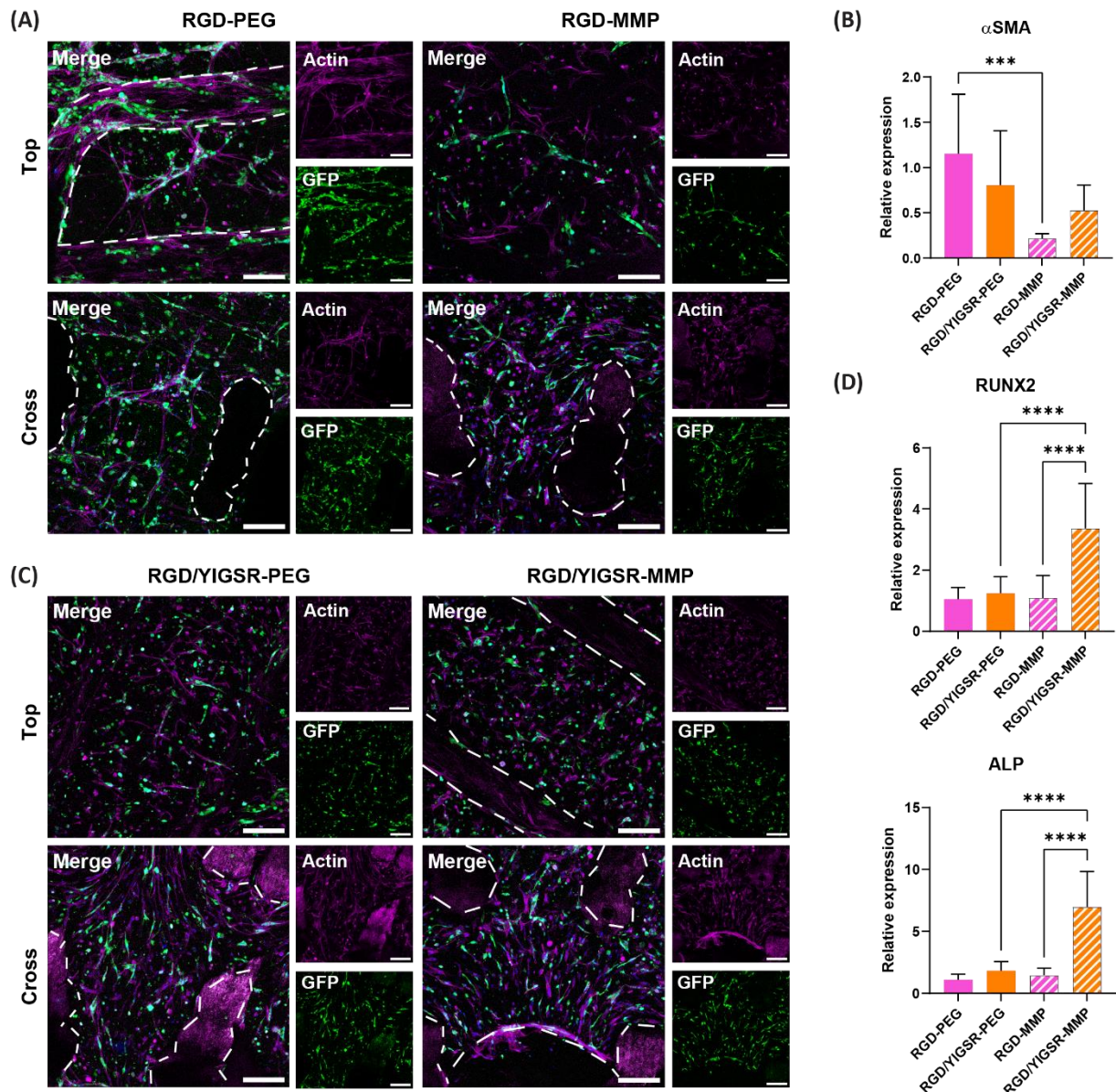


Figure 6. Assessment of cellular response in the hybrid constructs. (A) Fluorescent images (hUVEC-GFP=green, Phalloidin=magenta) of the co-culture condition in RGD-PEG or RGD-MMP hybrid scaffolds. Scale bar 200 μ m. (B) Relative gene expression of α SMA in the hybrid constructs with co-culture. Values normalized against the RGD-PEG condition. (C) Fluorescent images (hUVEC-GFP=green, Phalloidin=magenta) of the co-culture condition in RGD/YIGSR-PEG or RGD/YIGSR-MMP hybrid scaffolds. Scale bar 200 μ m. (D) Relative gene expression of RUNX2 and ALP in the hybrid constructs with co-culture. Values normalized against the RGD-PEG condition. White striped lines indicate the fibers of the scaffolds. Statistical significance represented by: ****<0.0001, ***<0.001.

Taken together, our results highlight that embedding the scaffold within hydrogels with different peptides and crosslinkers influences both endothelial organization and early osteogenic gene expression. Although no unique formulation seemed to simultaneously maximize both outcomes, the results provided insights into how the system can be tuned to guide specific cellular behavior. These effects can be attributed to the ECM-like component provided by the hydrogel, comprising chemical cues (e.g. peptides or crosslinkers) that can guide cell adhesion and matrix remodeling. Moreover, the composite

scaffold component provides mechanical and spatial cues to guide cells towards osteogenic differentiation [28,66,67]. However, differences in cell response were observed when comparing the different hydrogel chemical compositions. First, RGD constructs seemed to promote earlier and longer tubule formation, than the respective RGD/YIGSR scaffolds. We hypothesized that the higher RGD concentration in the RGD conditions (double than the mixed peptide) led to faster and greater cell adhesion to the hydrogel and subsequent matrix remodeling. Indeed, other studies demonstrated that cell adhesion to hydrogel matrices is highly dependent on RGD peptide concentration [68]. Instead, the RGD/YIGSR formulations appeared to be more involved in osteogenic commitment, particularly the MMP condition. This could result from a combined effect of lower concentration of adhesive peptide and matrix degradability. In fact, the MMP crosslinker is highly degradable and this could lead to greater migration of cells towards the scaffold fibers.

In the context of pre-vascularization, Matrigel® or GelMA have often been identified as benchmark hydrogels due to their inherent bioactivity and their well-documented ability to support vessel formation [69,70]. However, both present limitations that complicate systematic investigating of individual biochemical cues (e.g. peptides or crosslinkers), as well as their translational potential. Matrigel®, being animal-derived, presents high batch-to-batch variability and an undefined biochemical composition [71]. GelMA, despite being more controlled and characterized, lacks modularity, limiting for example the ability to independently control peptide functionalization or crosslinking chemistry. Therefore, the complexity of the elements that can be incorporated is limited. In contrast, the HA-based hydrogel developed in this study was specifically designed for precise control and tunability of matrix properties through peptide coupling or crosslinking. This provides a more defined and systematic platform to study cellular response to the different biochemical and mechanical cues. In our study, a direct comparison with Matrigel® or GelMA was not performed, as the scope of this study was not to develop a system that could outperform existing standard hydrogels. Furthermore, given the substantial differences in biochemical, mechanical and degradation properties, as well as crosslinking mechanisms, such a comparison would not allow a decoupling of individual properties.

Next to the importance of the matrix properties, our results also highlighted the importance of cellular crosstalk in guiding the tubule-like organization. While the hybrid scaffolds encapsulating monocultures of either hMSCs or hUVECs did not display any vessel formation, their co-culture instead promoted early cellular organization. These preliminary findings support the involvement of paracrine interactions between the two cell types. However, a deeper investigation of key pathways (e.g. VEGF, PDGF- β , PI3K/AKT) and their downstream factors could be performed in future studies, for instance through secretome analysis, to elucidate the molecular mechanisms involved in cell-cell communication within the hybrid scaffolds.

Ultimately, the goal is to implant a construct *in vivo* that can successfully support vessel inosculation and maturation and bone regeneration. In this study, we focused on discovering the pre-vascularization and osteogenic capacity of a novel hybrid construct from a more fundamental angle. Future studies should investigate complete cellular maturation and vascular network stability by performing longer cultures (e.g. 28 days), where expression of late osteogenic markers (e.g. Collagen I, Osteocalcin) and vascular maturation markers (e.g. Collagen IV, α SMA) could be assessed [72–74]. In addition, future research could focus on evaluating different cell seeding regimens, where endothelial and stromal cells are seeded sequentially, which was shown in the literature to be more effective in the maturation of the vascular network. This would better resemble the *in vivo* tissue repair dynamics, where first endothelial cells are activated and then, through the release of growth factors, stromal supportive cells are recruited [75,76].

Conclusion

In conclusion, this study demonstrated that hybrid scaffolds comprising an AM osteogenic scaffold and a peptide-functionalized HA hydrogel were able to support *in vitro* early vascular-like network formation and osteogenic commitment, when co-culturing endothelial and stromal cells. We successfully demonstrated that the biological outcome of the system can be controlled by tuning the chemistry of the hydrogel, in terms of grafted peptides and crosslinking sequence. Future studies will aim at validating these findings in longer cell culture studies and *in vivo* subcutaneous implantation in a mouse animal model to assess network maturation and inosculation capacity.

Acknowledgements

The authors would also like to acknowledge the European Research Council starting grant “Cell Hybridge” for financial support under the Horizon2020 framework program (Grant #637308). This project has also received funding from the European Union’s Horizon 2020 FET Open programme under grant agreement No. 964452 (BIRDIE project).

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CHAPTER 6

Supplementary information

Engineering a hybrid 3D construct for bone regeneration to promote simultaneous pre-vascularization and osteogenic differentiation in vitro

Sophia Dalfino, Sveva Fagiolino, Ivo Beeren, Marco Borrone, Francesco Alviano, Carlos Mota, Gianluca Tartaglia, Claudia Dolci, Lorenzo Moroni

Submitted to Advanced Healthcare Materials (Wiley)

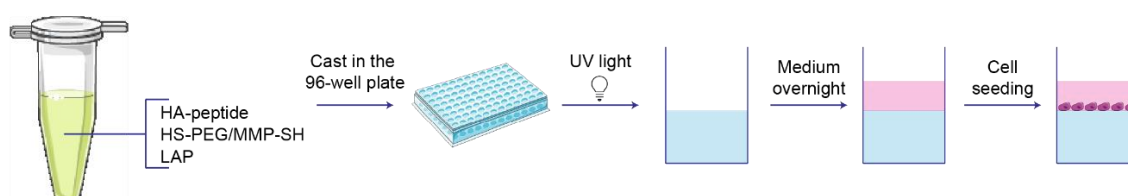
Table S1. List of antibodies and dilutions that were used in 2D and 3D experiments.

Antibody	Species & brand	Dilution
2D experiment		
Phalloidin	Alexa Fluor 488 - Thermofisher	1:500
Runx2	Rabbit – Abcam ab192256	1:300
CD31	Mouse – Abcam ab24590	1:400
Ve-Cad	Mouse – R&D systems af938	1:400
Seeding on top of the gels		
Phalloidin	Alexa Fluor 647 - Thermofisher	1:500
Encapsulation in the gels/hybrid constructs		
Phalloidin	Alexa Fluor 647 - Thermofisher	1:500

Table S2. Primer sequences of the genes, used for qPCR experiment: runt-related transcription factor (RUNX2), alkaline phosphatase (ALP), platelet endothelial cell adhesion molecule 1 (PECAM-1), kinase insert domain receptor (KDR), actin alpha 2 (ACTA2), beta-2-microglobulin (B2M, housekeeping gene).

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
RUNX2	TCAACGATCTGAGATTTGTGGG	GGGGAGGATTTGTGAAGACGG
ALP	ACAAGCACTCCCACCTTCATC	TTCAGCTCGTACTGCATGTC
PECAM1 (CD31)	GAAAGCCAAGGCCAAGCAGATG	TTTCCACGGCATCAGGGACAG
KDR (VEGFR2)	CCCTACAAGACCAAGGGGCAC	GCGATGCCAAGAAGTCCATGC
ACTA2 (aSMA)	ACGTGGGTGACGAAGCACAG	GGGCAACACGAAGCTCATTGTA
B2M	ACAAAGTCACATGGTTCACA	GACTTGTCTTTCAGCAAGGA

(A) Seeding on top of the gels



(B) Cell encapsulation in the hydrogel and in the hybrid constructs

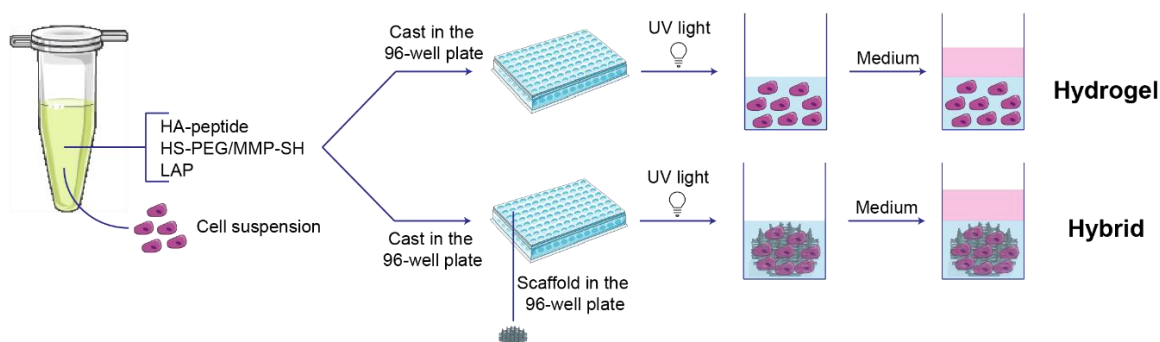


Figure S1. Schematics of the biological experiments conducted to validate the hydrogel and hybrid constructs. (A) Steps for the cell seeding on top of the gels. (B) Steps for the cell encapsulation in the hydrogel or in the hybrid constructs.

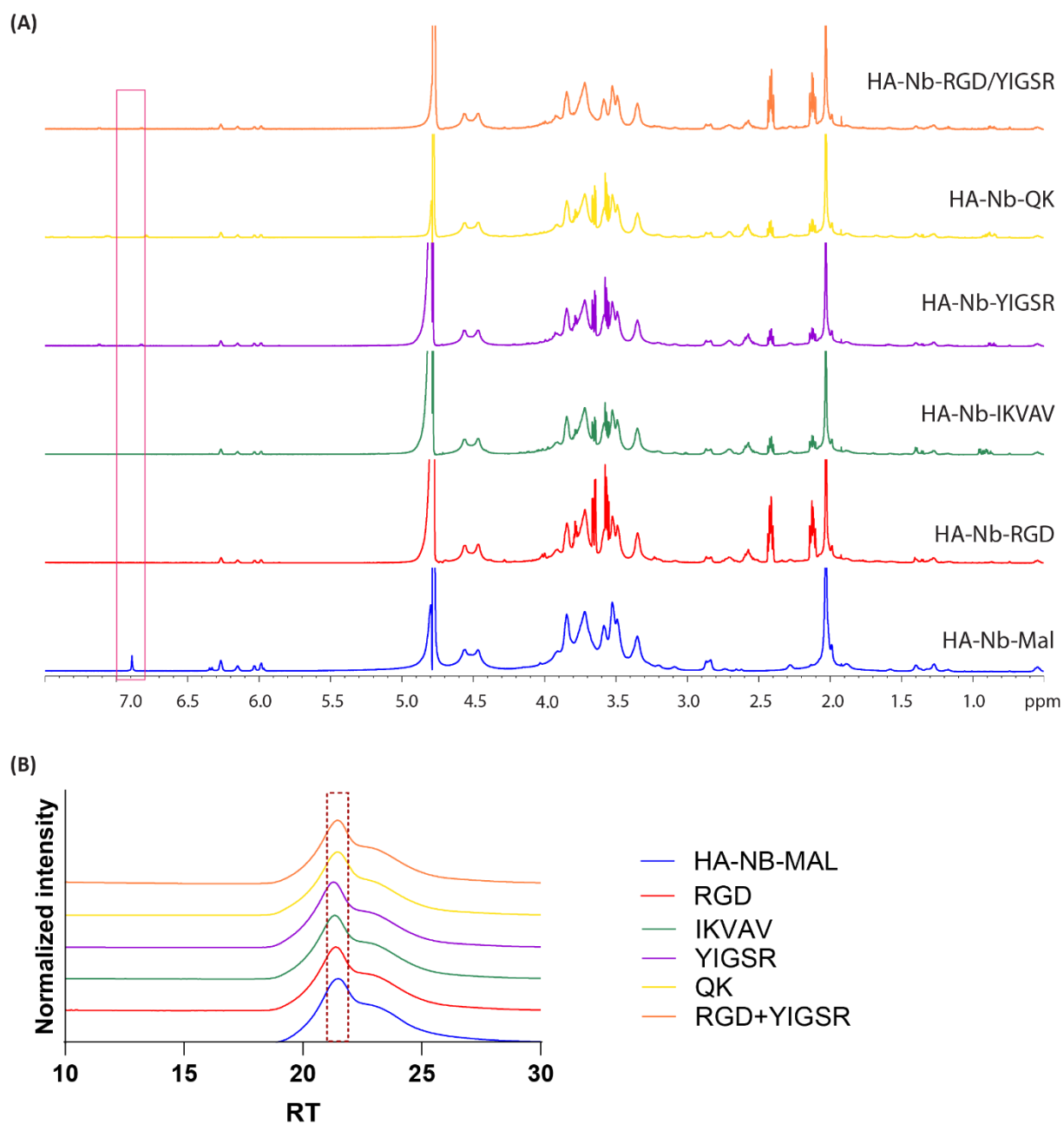
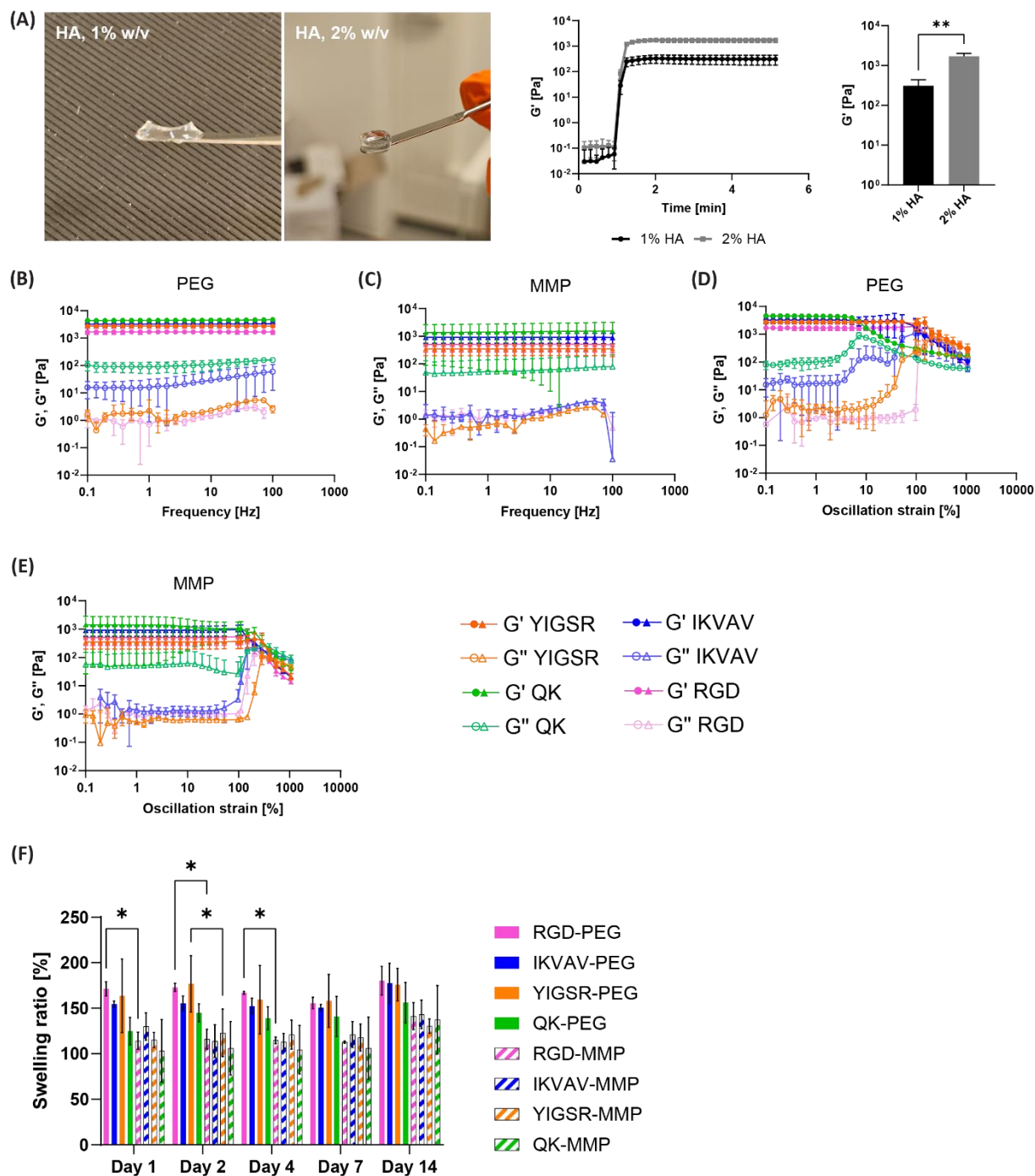


Figure S2. Characterization of HA after peptide functionalization. (A) NMR of the polymer after peptide grafting, showing that the Mal disappears. (B) GPC of the polymer with and without peptides, showing slight molecular weight increase when the peptides are grafted.



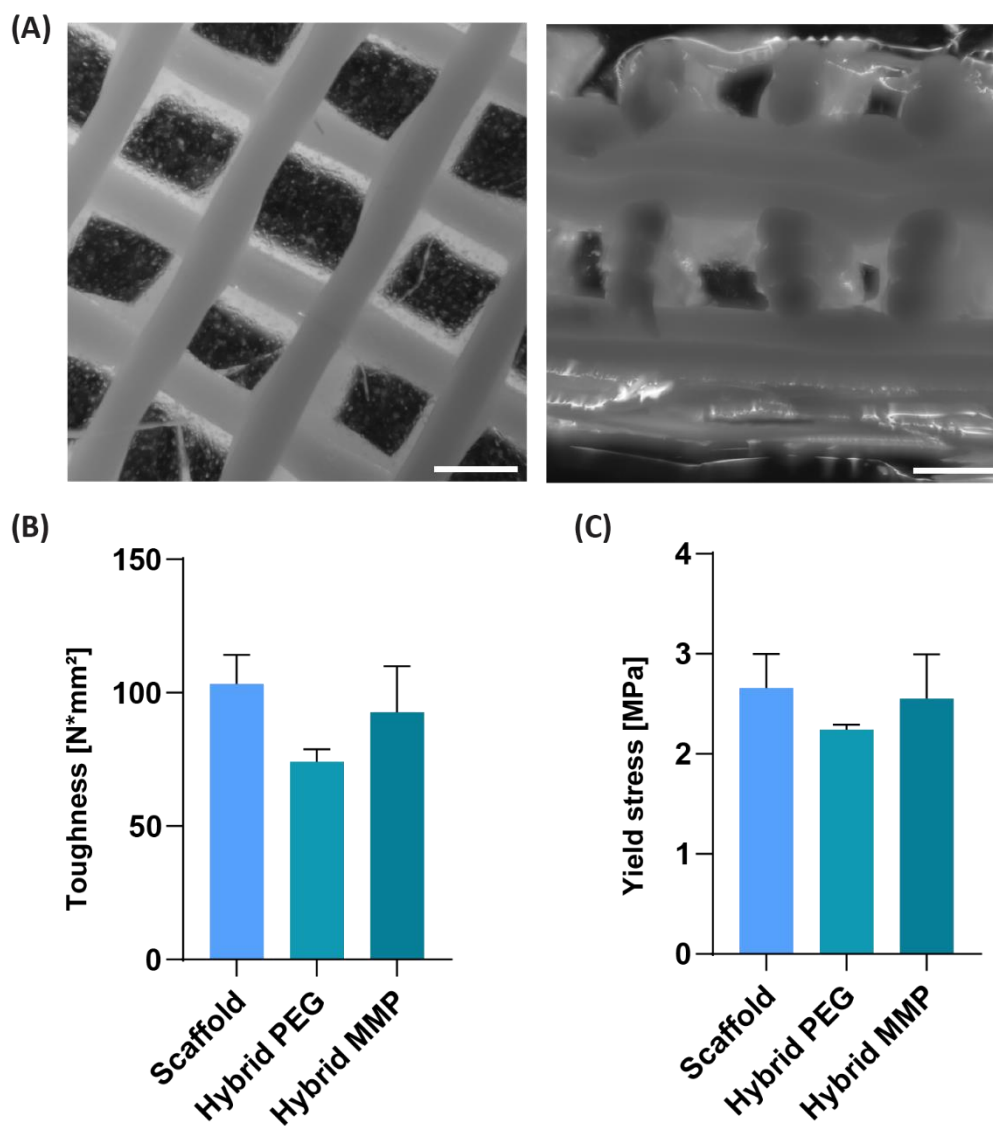


Figure S4. Characterization of hybrid constructs. (A) Stereomicroscope images of the hybrid constructs, comprising the AM scaffold and the HA hydrogel (top and cross-section view). Scale bar 500 μm . (B) Toughness values extracted from the stress-strain curves showing a comparison among different conditions. (C) Yield stress values extracted from the stress-strain curves showing a comparison among different conditions.

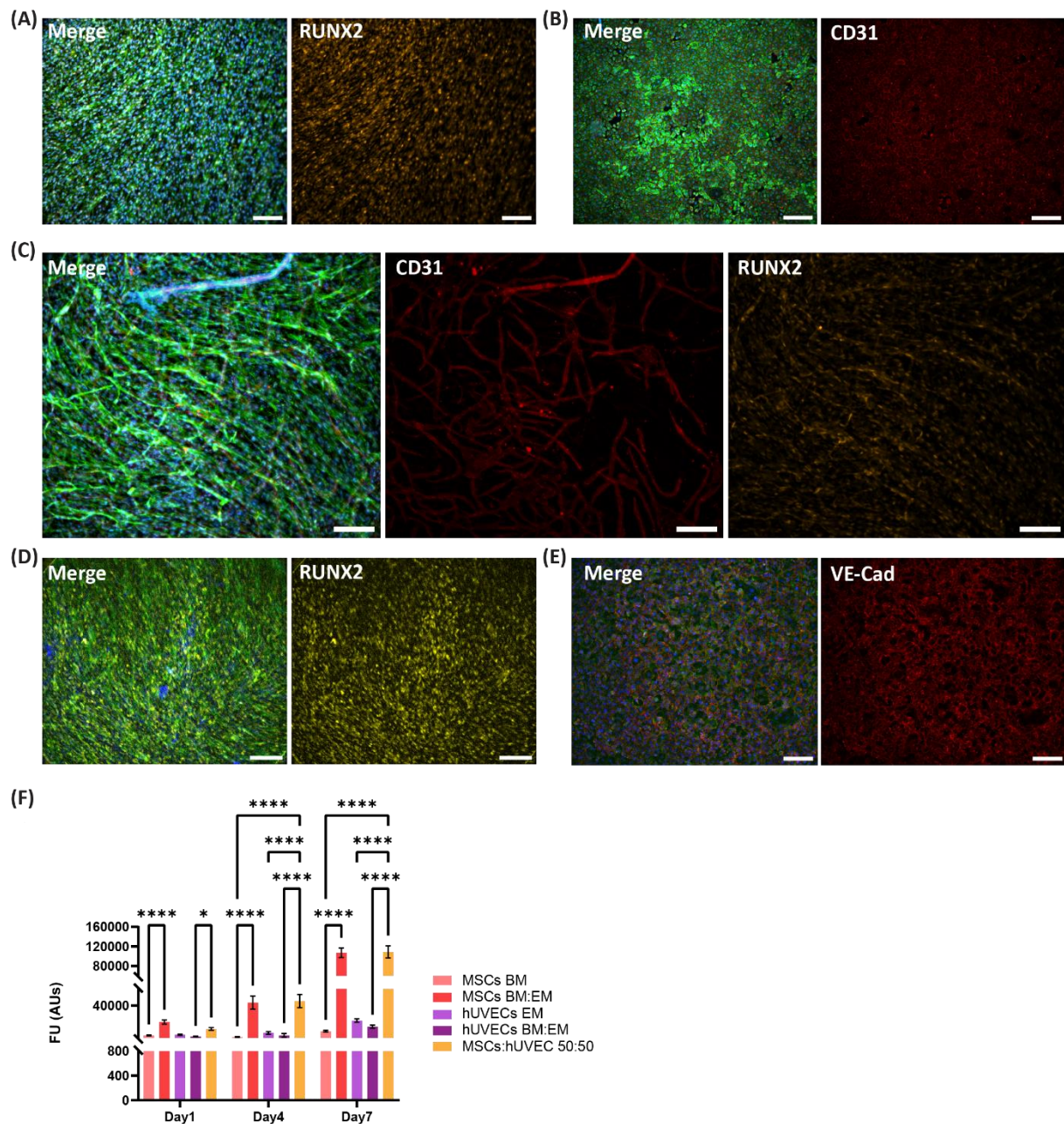


Figure S5. Immunostaining and metabolic activity of the 2D culture conditions of hMSCs and hUVECs alone or in co-culture, with different media conditions. (A) hMSCs after 7 days in BM:EM 50:50, positive for RUNX2. (B) hUVECs after 7 days in BM:EM 50:50, positive for CD31. (C) hMSC:hUVEC in a 1:1 ratio after 7 days in BM:EM 50:50, showing expression of both RUNX2 and CD31. (D) hMSCs after 7 days in BM only, positive for RUNX2. (E) hUVECs after 7 days in BM only, positive for VE-Cadherin. Scale bar = 200 μ m. (F) Metabolic activity measured with Presto Blue assay over 7 days for different culture conditions. Statistical significance represented by: **** <0.0001 , * <0.05 .

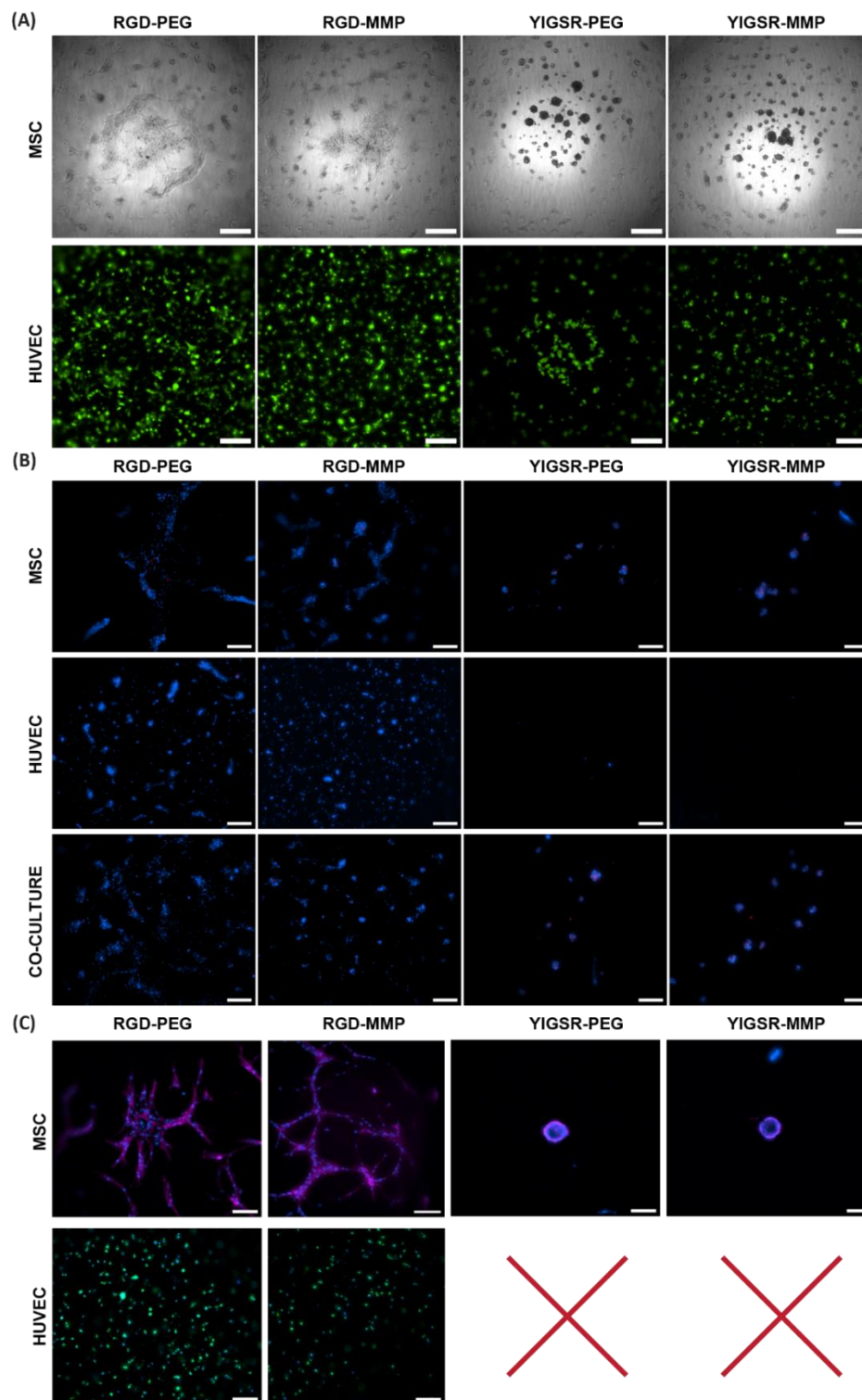


Figure S6. Assessment of the conditions of cells when seeded on top of the hydrogels. (A) Qualitative evaluation of the morphology of the hMSCs and hUVEC-GFP, cultured alone on each gel, after 24h from the seeding. Scale bar 200 μ m. (B) DAPI (blue) and dead (red) staining for all the culture conditions after 24h from the seeding. hUVECs on YIGSR conditions are missing because cells were washed away during staining. Scale bar 200 μ m. (C) Immunostaining of the hMSCs and hUVECs after 7 days of culture on different gel conditions. DAPI (blue), Phalloidin (magenta), hUVECs-GFP (green). No hUVECs left on the YIGSR gels after 7 days, due to washing steps involved in the staining procedure. Scale bar 200 μ m.

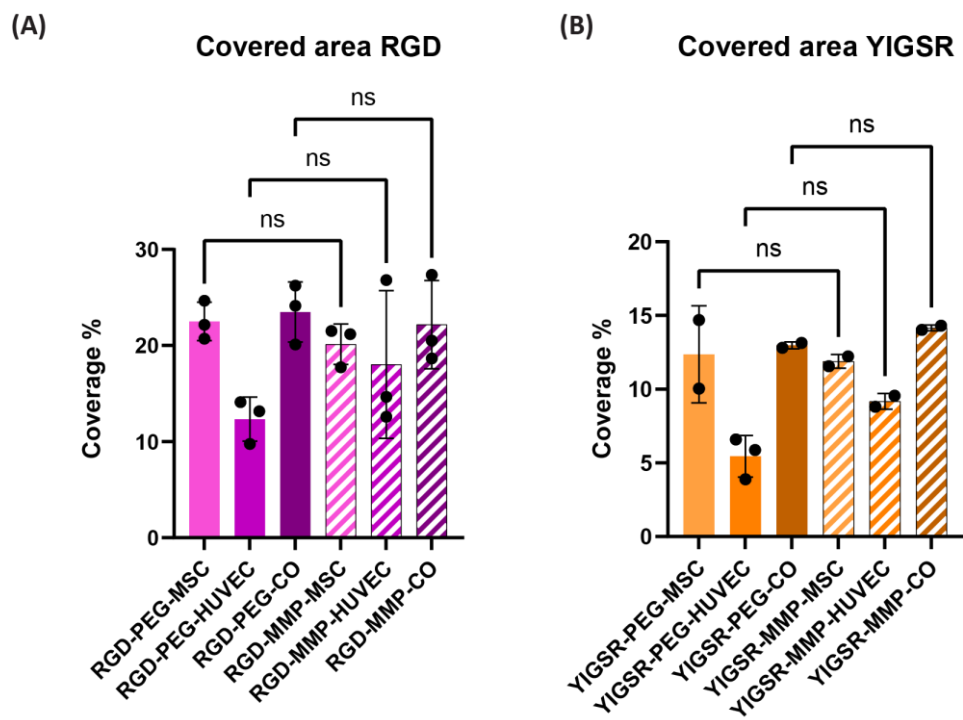


Figure S7. Assessment of cell response when seeded on top of HA-RGD-PEG/MMP or HA-YIGSR-PEG/MMP hydrogels. Coverage% of the cells seeded on top of the RGD (A) and YIGSR (B) gels. Statistical significance represented by: ns > 0.05.

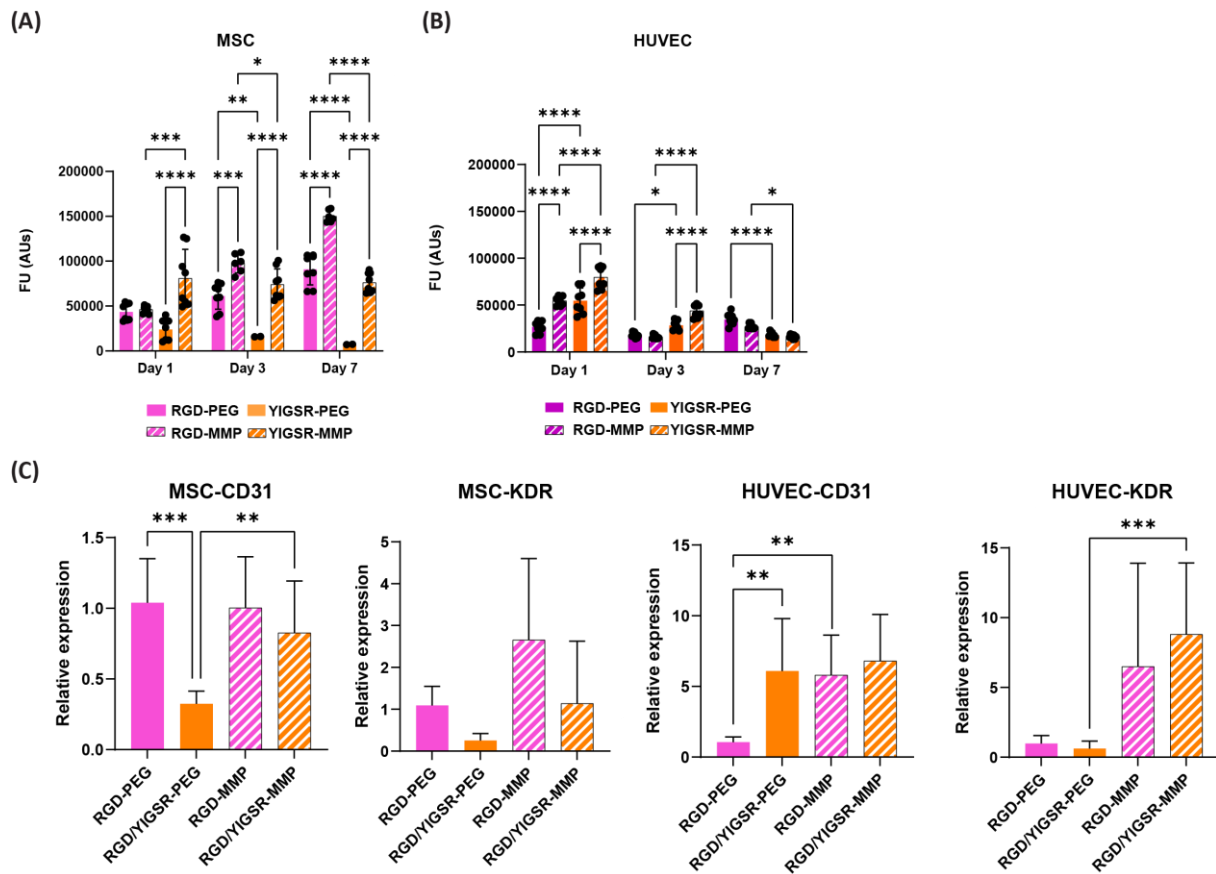


Figure S8. Assessment of the conditions of cells encapsulated in different hydrogel formulations. Metabolic activity over 7 days of (A) hMSCs and (B) hUVECs. (C) Gene expression of CD31 and KDR genes in the hydrogels only for hMSCs and hUVECs. Values normalized against the RGD-PEG condition. Statistical significance represented by: ****<0.0001, ***<0.001, **<0.01, *<0.05.

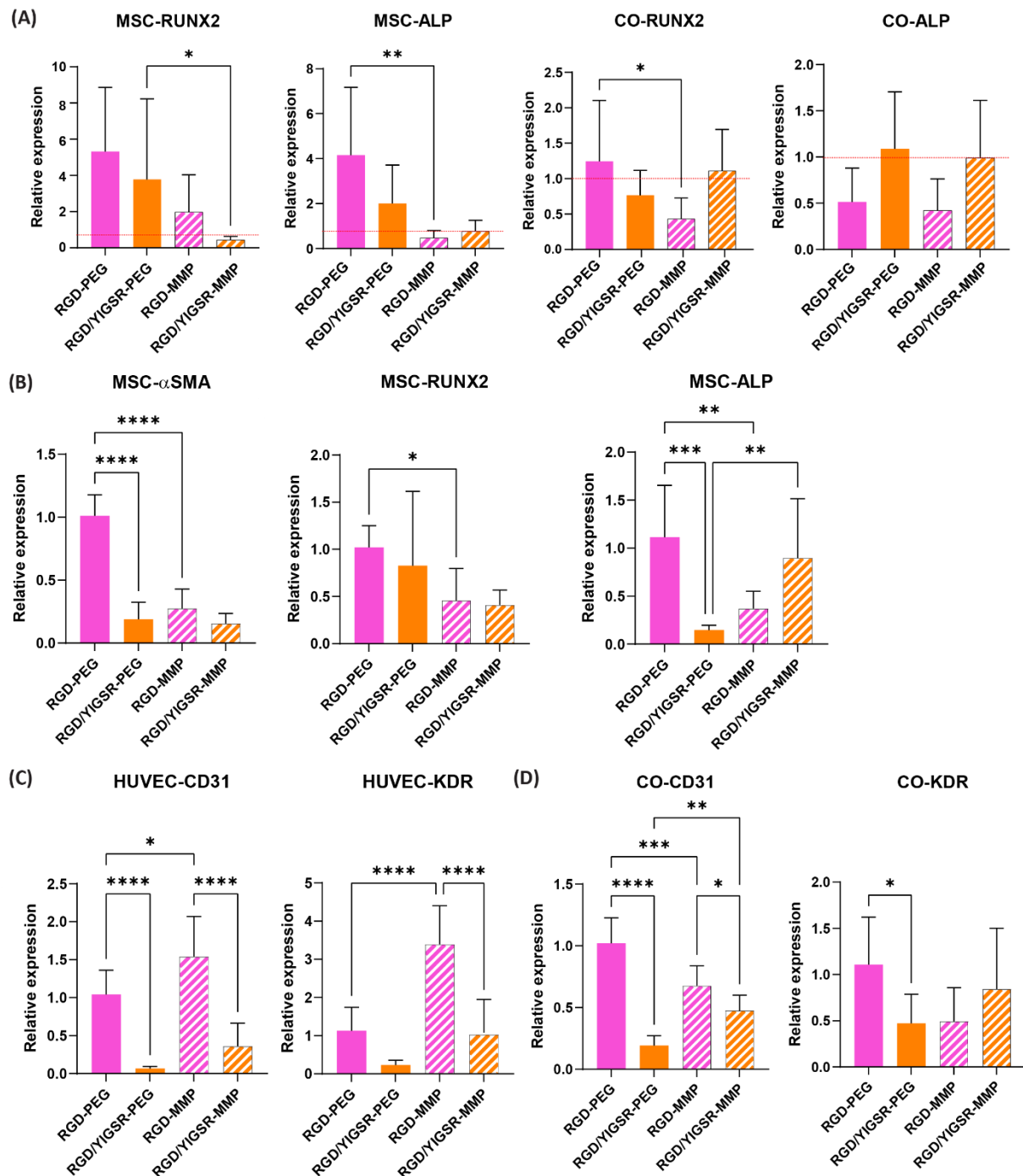


Figure S9. Assessment of the conditions of cells encapsulated in different hybrid construct formulations. (A) Gene expression of RUNX2 and ALP in the hybrid constructs normalized against the respective hydrogel only conditions, showing that the scaffolds upregulated osteogenic gene expression for hMSCs and co-culture conditions. (B) Gene expression of α SMA, RUNX2 and ALP in hMSCs monocultures in hybrid constructs normalized against the RGD-PEG condition, showing early osteogenic markers in hMSCs. (C) Gene expression of CD31 and KDR in hUVECs monocultures in hybrid constructs normalized against the RGD-PEG condition, showing endothelial markers in hUVECs. (D) Gene expression of CD31 and KDR in the co-culture conditions in hybrid constructs normalized against the RGD-PEG condition, showing endothelial markers in co-culture condition. Statistical significance represented by: ****<0.0001, ***<0.001, **<0.01, *<0.05.

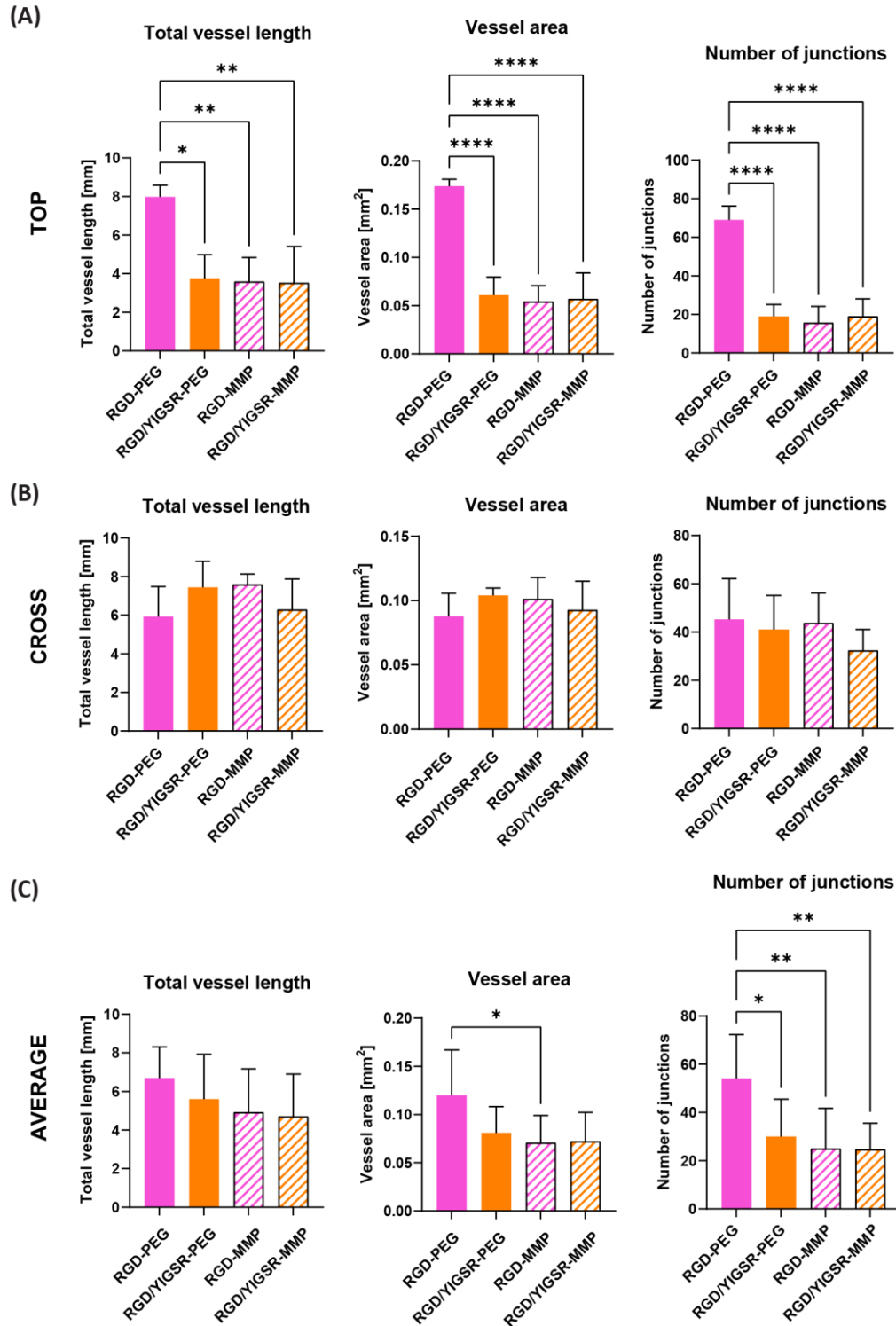


Figure S10. Quantification of fluorescent images in the co-culture conditions to assess vessel formation parameters after 7 days of culture. Total vessel length, vessel area and number of junctions were quantified on confocal images taken both from the top (A) and from the cross section (B) of at least 3 replicates per condition. An average of top and cross section results was also calculated for a better estimation of the results (C). Statistical significance represented by: ****<0.0001, **<0.01, *<0.05.

CHAPTER 7

General discussion

Introduction

Bone tissue engineering (BTE) aims at restoring damaged tissue by combining biomaterials, cells and bioactive factors into a 3D scaffold. Scaffolds are typically designed to resemble features of the native tissue in terms of material and geometry to support cell adhesion, proliferation and differentiation, guiding new tissue formation.

Mandibular BTE represents a promising alternative to autologous bone grafts, the current gold standard treatment for mandibular defects, by enabling patient-specific constructs that support bone regeneration. Over the past decades, some biomaterial-based solutions have progressed to the clinics for bone regeneration, such as biphasic calcium phosphate [1], tricalcium phosphate-based (e.g. MagnetOs™ from Kuros Biosciences, Osteogrow-C [2]) or bioactive glass granules (e.g. Bonalive®) used *in vivo* for long bone or craniomaxillofacial reconstruction. All these solutions are mostly applied to treat small non-critical defects and remain not suited for complex mandible defects. Importantly, since these biomaterials are applied in their bulk form, there is no control over pore geometry and macro architecture, hindering mandible applications. In this context, additive manufacturing (AM) technologies emerged as tools to fabricate complex and customized scaffolds, as they offer the possibility to control macro- and micro- architecture and to process a broad range of biomaterials.

Limitations in clinical translation of AM scaffolds

Commercially available AM scaffolds, such as those developed by Osteopore or Finceramica, have demonstrated the technical and clinical feasibility for craniofacial and mandibular applications. For example, polycaprolactone (PCL) or composite PCL/ β -tricalcium phosphate (β -TCP) scaffolds have been successfully applied in long bone [3] and mandibular defects [4], while hydroxyapatite (HA) scaffolds have promoted osteointegration and bone formation in skull defects [5]. However, in most clinical applications these scaffolds are part of a combined surgical strategy, for instance in combination with vascularized autologous flap, aspirate bone marrow, platelet-rich plasma or growth factors.

Insufficient vascularization is the most critical challenge for AM scaffolds, as vessel growth and subsequent bone formation is often limited to scaffold periphery [5,6]. This is also associated with incomplete defect bridging that compromises both functionality and mechanical stability [7]. In addition, scaffolds often exhibit suboptimal mechanical properties, which make them not suited to withstand the high and cyclic loading of the mandible [8]. In some cases, immune responses triggered by scaffold implantation can lead to fibrotic tissue formation, impairing integration with the native tissue [9]. Furthermore, a lack of standardization in preclinical and clinical protocols, including

differences in defect size, animal models and parameters to evaluate, complicate the reliable assessment and comparison of results across studies [10].

In **Chapter 2** of this thesis, we reviewed the main limitations and requirements of scaffolds for mandibular regeneration, with particular focus on AM approaches. We outlined the main scaffold features that influence osteogenesis and angiogenesis (e.g. scaffold geometry and intrinsic biomaterial properties). We also discussed the lack of standardization in preclinical and clinical studies as another barrier to translation. From this literature analysis, we identified the main points to address in the thesis: improving osteogenic and angiogenic performance of scaffolds through different strategies.

Enhancing osteogenesis and angiogenesis: 4 strategies

Osteogenesis and angiogenesis are influenced by a variety of intrinsic properties of AM scaffolds, such as morphology, material, and mechanical properties [9,11]. In addition, other bioactive components can be added to the scaffold after fabrication to pivot the bioactive characteristics. In this thesis, we first explored the effect of different scaffold pore morphologies and the addition of inorganic materials on osteogenic or angiogenic processes in human mesenchymal stromal cells (hMSCs). We then moved to more complex strategies, creating new small molecule release systems and cell-based approaches within the constructs.

The role of morphological properties in enhancing osteogenic processes

The term “morphological properties” refers to all design parameters that determine the final architecture of the scaffold. These properties, including bulk shape, pore geometry and size, total porosity and pore interconnectivity, can be tuned during the design stage prior to fabrication. Pore size and total porosity have been acknowledged as one of the parameters influencing bone formation. In this thesis, scaffolds were designed with an *in vivo* application in mind. Therefore, we adopted a scaffold porosity >70% and a pore size >900 μm across all chapters, as large porosities (>70%) and pore sizes (>600 μm), combined with interconnected pores, facilitate nutrient and oxygen diffusion, vascularization and bone formation *in vivo* [12–14].

Pore morphology is another parameter that was shown to be connected to osteogenic differentiation in bone tissue engineering applications. Initial AM scaffolds adopted woodpile architecture, consisting of a 90° rotation between layers with a continuous interconnected rectangular pore network, because of its simplicity and ease of fabrication. However, more recent advances demonstrated that changes of angle between layers or more biomimetic geometries could be beneficial for osteogenic marker expressions [15,16]. This behavior is associated with the role of curvature in guiding tissue regeneration processes [17,18]. Concave curved surfaces and more acute angles between

fibers were demonstrated to be more successful in promoting cell adhesion, cytoskeleton organization and osteogenic differentiation [19–23].

Based on these findings, in **Chapter 3** we investigated two pore geometries: a “star” design, obtained by a 0-15° interlayer rotation, previously shown to enhance osteogenic markers such as ALP [20], and a diamond triply periodic minimal surface (TPMS), which is particularly attractive for its surface curvatures and biomimicry of trabecular bone architecture [24,25]. We fabricated AM scaffolds with complex pore geometries in two different materials, PCL only or a composite of PCL/ β -TCP. The geometries were compared for their biological and mechanical behavior. Different effects were observed in the osteogenic differentiation of hMSCs seeded on the two scaffold types: the diamond geometry favored overall osteogenic marker expression, such as ALP activity or collagen deposition, which we attributed to the higher biomimicry of this design [26]. Instead, the star geometry enhanced mineralization in the PCL/ β -TCP condition, as indicated by higher calcium deposition measured with Alizarin Red staining. This was likely due to the larger scaffold surface area implying greater exposure of ceramic particles [26]. From a mechanical perspective, diamond scaffolds exhibited greater compressive moduli compared to star-shaped constructs, weakened by the lower amount of contact points between consecutive layers [20,26,27].

Our research has been in line with recent studies, who investigate and demonstrate repeatedly the superiority of unconventional pore geometries over traditional woodpile-shaped scaffolds in terms of bone mimicry. Among these, TPMS scaffolds have emerged for their osteogenic potential both *in vitro* and *in vivo*, mostly attributed to curvature-driven cell migration and guidance (curvotaxis) [28–31]. Similarly, Voronoi tessellation has been reported for its biomimetic architecture [32,33], with 3D Voronoi scaffolds achieving permeability values comparable to cancellous bone [34]. Composite PCL/hydroxyapatite (HA) Voronoi scaffolds were shown to promote vascularized tissue formation without fibrotic encapsulation in a rat ectopic model [35]. In line with this, Channasanon et al. reinforced the importance of geometry by directly comparing a woodpile scaffold and two different pore sizes of a more complex four-coordinate scaffold [36]. The study showed that in a dynamic bioreactor, the larger-pore complex design promoted higher osteogenic gene expression, whereas the smaller pores (with higher surface area) favored mineral deposition, similarly to what observed in our study. Computational fluid dynamic (CFD) simulations provided a mechanistic explanation, showing that larger pores improved nutrient and oxygen flow, thereby stimulating differentiation [36].

Fluid dynamics and mechanical properties likely explain the different biological responses observed across scaffold geometries. In our study, differences between the two geometries may have been partially masked by the static culture conditions. We hypothesized that under dynamic stimulation, variations in geometry would result in

different distributions of mechanical load and fluid flow. Supporting this, Rubert et al. compared cubic and spherical unit cell-based scaffold in both static and dynamic conditions, showing that differences in the wall shear stress between the two geometries resulted in greater differences between geometries in dynamic cultures [37]. Similarly, differences in mechanical properties, such as those we identified between star and diamond scaffolds, could influence biological outcomes in dynamic compression settings. Indeed, scaffolds with lower stiffness are expected to undergo higher strain, which could induce different mechanical cues sensed by cells [38]. Taken together, future work could consider applying dynamic culture systems (e.g. bioreactors with medium flow or bioreactors for cyclic compression) to better recapitulate *in vivo* conditions and assess the effects of the two geometries we investigated [39].

While our findings highlighted the osteogenic potential of diamond shaped scaffolds, we identified a potential shortcoming with this approach related to the selected printing technique. We fabricated diamond scaffolds with melt extrusion-based AM, which relies on a layer-by-layer approach that cannot fully capture the 3D complexity of native TPMS. This geometry, as also visible in micro-CT analysis, presented a more 2.5D diamond network rather than a 3D complexity. Since curvature, as mentioned above, is an important cue for guiding osteogenic differentiation, this reduced shape fidelity may have partially compromised the osteogenic potential of the design. Alternative manufacturing strategies such as light-based technologies (e.g. DLP, volumetric printing) were reported to be applied to TPMS structures fabrication and could be explored in future studies to overcome the presented limitations [40–44].

Leveraging material intrinsic properties to enhance osteogenesis and angiogenesis

In addition to morphology, scaffold biomaterials are central in modulating cell responses via their intrinsic molecular composition and mechanical properties. Due to the high mineral content of bone (around 70% of its dry weight), the addition of different inorganic ceramics into a polymeric matrix has been widely adopted for BTE applications. In **Chapter 3** and **Chapter 4**, we followed this approach by blending different ceramic components with a medical grade PCL to produce composite materials suitable for melt extrusion-based printing. Next to increasing polymer bioactivity, this strategy also allowed the processing of the ceramic material in more friendly conditions, involving lower temperatures and avoiding the use of potentially toxic binders [45]. In addition, blending a ceramic and a polymeric component allows to improve the final mechanical properties of the constructs that presents a plastic behavior, without compromising the scaffold architecture and porosity [46–48].

In **Chapter 3**, next to investigating different pore geometries, we produced a composite of PCL/ β -TCP. β -TCP contains Ca and P ions, which are also highly present in the mineral

phase of native bone [49]. This similarity and the release of Ca and P ions contribute to enhance osteo-inductive and -conductive properties of the material, promoting osteogenic differentiation and matrix mineralization [50–53]. In our study, we observed delayed activation of the osteogenic differentiation cascade, with minor differences in osteogenic genes (e.g. early genes RUNX2 and ALP as well as late genes BMP2 and COL1) between the composite and pristine PCL scaffolds within the first 28 days. Notably, a significant effect was only observed on mineralization after 28 days of culture. We hypothesized that the ceramic material was fully encapsulated after scaffold fabrication and covered by a thin polymer layer, as also reported in previous studies [54,55]. As a result, the exchange of osteo-inductive ions with the media was delayed as we observed in release studies performed using spectrometric techniques (ICP-MS), where Ca and P ions concentrations were comparable between the composites and the blank release media. Only after 28 days of scaffold incubation, scanning electron microscopy (SEM) imaging revealed exposure of the particles, implying that polymer degradation likely occurred at later time points. Therefore, in the next chapter, we introduced a sodium hydroxide (NaOH) surface treatment, a strategy reported to degrade the outer surface layer prior to cell seeding [56,57].

In **Chapter 4**, we selected bioactive glasses (BGs) as ceramic components to blend with PCL. They are usually composed of Ca and P, together with other biologically relevant ions, such as Si, Na or Mg [58,59]. The presence of these ions, specifically of Si, gives to BGs not only osteogenic but also angiogenic properties [60–62]. In our study, we selected two novel BGs produced starting from two different layers of the same source of sand. The two glasses have similar macro composition, mainly composed of Si, Ca and Na ions, but they differ for the trace elements, and this might be associated with different biological outcomes. A previous *in vitro* and *in ovo* study on the raw BG powders showed their high osteogenic and angiogenic potential [63]. Thus, in our study, we blended the BGs with PCL, we fabricated AM scaffolds and applied a NaOH surface treatment. This resulted in a burst release of Si and Ca ions measured in the first 24h of scaffold incubation and a sustained release over 28 days. As a consequence of the improved ion release, a significant upregulation of early osteogenic gene RUNX2 occurred after 7 days of culture, while late osteogenic genes COL1 and OCN were upregulated at later time points on the composite scaffolds compared to PCL alone. In addition, the composite scaffolds containing BGs displayed angiogenic effect, by inducing the early upregulation of VEGF gene, and secretion of pro-angiogenic factors (e.g. VEGF or CCL2), which also enhanced tubule formation on endothelial cells. Based on these promising results, future studies could aim at integrating a direct co-culture of endothelial cells and hMSCs on the PCL/BGs scaffolds to assess whether endothelial network formation would occur.

The use of ceramics in BTE has progressively evolved. Traditionally, hydroxyapatite (HA) has been the most used ceramic, showing improved bioactivity and osteogenic cell differentiation [64]. However, due to its slow degradation limiting bioactive properties, it has been slowly replaced by other ceramic fillers, such as TCP or biphasic calcium phosphates (BCP) [65]. Consistent with our first study, several groups have reported the success of these composite scaffolds in enhancing osteogenic differentiation *in vitro* and increased bone formation *in vivo* [66–69]. While these types of composite scaffolds demonstrated osteogenic potential, they were often limited in their ability to stimulate vascularization. More recently, attention has shifted toward dual-function ceramics able of simultaneously promoting osteogenesis and angiogenesis. In this context, BGs have emerged for their versatility and, in agreement with our findings, multiple studies have highlighted their promising osteogenic and angiogenic potential [70–73].

Building on this, numerous groups have explored an alternative strategy to enhance the biological performance of ceramic-based scaffolds through ion doping. For instance, the incorporation of magnesium, strontium, cobalt or zinc into calcium phosphates [74–76] or calcium silicate ceramics [77,78] has been shown to accelerate ion release and significantly enhance both osteogenic differentiation and angiogenesis compared to ion-free composite scaffolds. Considering these findings, ion doping represents a promising avenue for future development of this thesis, where the introduction of selected bioactive ions into the PCL/ β -TCP or PCL/BG scaffolds could further boost angiogenic capacity.

A recurrent limiting factor across studies on ceramic-based composite scaffolds is the achievable ceramic content, which is strongly connected with the biological outcomes. Higher ceramic loadings increase the available bioactive surface area and ion release, yet they also introduce a trade-off with printability and mechanical properties. Typical ceramic amounts were reported in a range of 10-50% w/w relative to the polymeric component [10]. In this thesis, we achieved up to 40% w/v β -TCP and 20% w/v of each BGs, beyond which printability was impaired by increased viscosity, and reduced mechanical integrity due to brittleness. Interestingly, using a different commercial BG (Bonalive®, not detailed in this thesis), we reached up to 50% w/v loading, implying that the maximum threshold is highly dependent on glass chemistry.

To overcome these constraints, some groups have focused on developing ceramic-containing composites suited for light-based printing technologies. For example, Das et al. printed a slurry containing 60% of HA/BGs, achieving high compressive strength and cell proliferation [79]. Similarly, Liu et al. produced low viscosity slurry by mixing 60% of TCP content with acrylate resin for DLP fabricated scaffolds [80]. These advances suggest that combining higher ceramic content with alternative printing approaches could allow the fabrication of scaffolds that better balance bioactivity, mechanical properties, and printability.

Introducing surface coatings to speed up osteogenic and angiogenic responses

The results described in Chapter 4 demonstrated that surface treatment improved particle exposure, accelerating the differentiation response to 7 days compared to the 28 days observed in Chapter 3. The NaOH etching, however, is known to compromise the mechanical integrity of the scaffolds [81,82]. By optimizing treatment time and solution concentration, we minimized the brittleness, yet surface cracks remained superficial. As result of this trade-off, we believe that cell exposure to ceramic particles is limited: most particles are still embedded within the polymer matrix and unlocking their bioactivity depends on the slow degradation of PCL.

To overcome these limitations, we next explored surface coating as a strategy to further enhance the speed and strength of the osteogenic and angiogenic response. Surface coatings provide immediate accessible physical and biochemical cues to cells [83,84]. For example, different studies reported the use of plasma treatments [85,86] or polydopamine [87,88] coatings to improve the adhesive properties of synthetic polymers. However, these solutions allow for adhesion of multiple cell types without specificity, limiting control over the activation of specific differentiation pathways [86,89]. Calcium phosphate/silicate-based [90,91] or peptide-based [92–94] coatings have also been applied for BTE to induce osteogenic or angiogenic differentiation. These approaches are often associated with drawbacks. Ceramic coatings were reported to have limited stability and, in some cases, poor adhesion to the underlying substrate [95]. Peptide coatings are sometimes associated with low grafting efficiency [94].

In **Chapter 5**, we explored an alternative strategy based on a drug-loaded bioactive coating on the scaffold fibers. We proposed an elastin-like recombinamer (ELR) coating able to self-assemble upon crosslinking, forming a stable and tunable coating [96]. The ELR sequence was modified with a statherin domain, which was shown to promote mineralization by nucleating CaP deposition and possess intrinsic osteogenic properties [96,97]. Such coatings were used for different applications such as polymeric scaffolds [97], metal surfaces [98] or as membranes to create functional topographies [99]. In our study, we coated PCL/ β -TCP scaffold fibers with ELRs to combine two complementary strategies: the sustained bioactivity provided by ceramic filler release and the immediate biological effect of the coating. In addition, we leveraged the coating as a release platform for two pro-angiogenic small molecules, deferoxamine (DFO) or 1,10-phenanthroline (Phen), aiming to trigger a rapid, simultaneous angiogenic and osteogenic response [100,101].

When we measured the release profile of the molecules, we observed that both displayed a burst release in the first 24h, with cumulative release of around 96% for Phen and 45% for DFO. We attributed this phenomenon to the small size of the molecules that quickly diffused out of the coating in which they were only physically adsorbed. In 2D tests

where we exposed hMSCs to several drug concentrations, we demonstrated that both molecules were able to induce hypoxic and pro-angiogenic response within 24h of incubation, as confirmed by the upregulation of VEGF and the nuclear translocation of HIF-1 α . Furthermore, after 10 days of culture, scaffolds releasing specific concentrations of the molecules showed significant upregulation of osteogenic genes (RUNX2, BMP2, ALP, SP7), demonstrating that the combination of the osteogenic coating and angiogenic factors synergistically enhanced biological response. Overall, these results indicate that our system effectively promoted an early and coordinated angiogenic-osteogenic effect. While this early effect is promising, longer culture times would be necessary to evaluate tissue maturation.

Our study presented for the first time the use of an ELR coating as a platform to deliver pro-angiogenic molecules from AM scaffolds. Previous applications of ELRs in BTE have primarily focused on improving osteogenesis, either as coating [102], hydrogel [103] or as free-standing membrane [104], or angiogenesis through endothelial cell-attracting peptide coupling [105]. For instance, Vila et al. fabricated an ELR-coated Si-doped HA scaffold demonstrating successful differentiation of hMSCs *in vitro* [106]. Interestingly, in most of these studies, ELR coating was also pre-mineralized to allow crystal formation prior to cell seeding or *in vivo* implantation, with the hypothesis that this would further enhance osteogenic processes. While we did not pursue pre-mineralization in our work, this could be an interesting future step for our ELR-coated scaffolds.

This thesis also introduced for the first time the release of Phen from osteogenic scaffolds as a strategy to enhance angiogenesis. To our knowledge, Phen has not been previously incorporated into 3D scaffold systems and its use in BTE has been limited to 2D cultures. In contrast, DFO has been mostly employed as hypoxia-mimicking drug, together with hypoxic ions (e.g. cobalt) [107] or dimethyloxallylglycine (DMOG) [108]. Typically, these molecules are released from the AM scaffolds via encapsulation in hydrogel coating, nanoparticles or are covalently grafted to the scaffold surface. DFO-loaded GelMA hydrogel was used to coat a 3D composite scaffold resulting in a sustained drug release that ensured osteogenic and angiogenic effect *in vitro* and improved osteointegration *in vivo* [109]. Beyond bone applications, PCL electrospun scaffolds were coated with DFO-loaded GelMA hydrogel favoring angiogenesis for chronic wound regeneration [110].

These studies mostly reported a sustained release of the drug over time. On the other hand, the work of this thesis showed a quick burst release of Phen within the first hours. Despite proving the efficacy of our system, we identified this as a potential limitation in view of an *in vivo* application, since it may be suboptimal to support all stages of bone and vessel formation. Future studies could aim at hybrid release systems that combine an immediate release of part of the therapeutic agent to initiate early angiogenic and osteogenic pathways, with a sustained release component achieved through covalent

immobilization or nanoparticle carriers. Such dual approach has already been shown to be promising in promoting sequential tissue regeneration [111–113] and could be optimized on the ELR-coated scaffolds.

Pre-vascularization to achieve in vitro micro-vessel formation

In Chapter 3, 4 and 5, we have discussed strategies to promote both osteogenesis and angiogenesis by directly modifying thermoplastic scaffolds. These strategies rely on the body's intrinsic capacity to colonize and vascularize the implant. While effective, this process takes time: even though angiogenic and osteogenic genes are rapidly activated, cells still need to migrate into the scaffold, proliferate, deposit extracellular matrix, and establish a vascular network. To address this intrinsic delay, we introduced in **Chapter 6** an *in vitro* pre-vascularization as an alternative strategy, in which a microvascular network is established prior to implantation [114]. This approach enables a more rapid integration with host vasculature *in vivo*, thereby accelerating perfusion and bone regeneration [115,116].

In our work, we presented an *in vitro* pre-vascularization strategy by developing a hybrid 3D construct combining a hydrogel and an AM scaffold, where endothelial and stromal cells were co-cultured for 7 days. This system takes advantage of the osteogenic potential of PCL/ β -TCP composite scaffolds with diamond pore geometry developed in Chapter 3, together with the extracellular matrix-mimicking properties of a hyaluronic acid-based (HA) hydrogel. The purpose of this study was to fundamentally investigate how different hydrogel chemistries influence early cellular response. To this end, two functional groups were introduced on the HA backbone, maleimide and norbornene. HA was functionalized with different peptides (e.g. RGD, YIGSR, or a combination of the two), via thiol-Michael addition. Hydrogel crosslinking occurred on norbornene units, through two crosslinkers (PEG-diSH or MMP-diSH). We produced hydrogels with different biochemical cues for adhesion and angiogenesis, as well as mechanical properties. We encapsulated hMSCs, HUVECs, and a co-culture thereof in the hydrogel to study the capacity of the hybrid construct to induce tubule formation as well as osteogenesis. The co-culture of endothelial cells and hMSCs was essential to ensure tubule-like structure formation throughout the scaffolds, independently of the hydrogel formulation. This supported our hypothesis that cellular crosstalk is key in regulating the coupled osteogenic and angiogenic processes [117,118]. However, the co-cultures responded differently to the tested hydrogel formulations. For instance, RGD-modified hybrid scaffolds promoted longer tubes and a pericyte-like behavior of hMSCs with upregulation of α SMA gene. In contrast, the RGD/YIGSR combination favored a more osteogenic behavior, with significant upregulation of RUNX2 and ALP. We attributed this shift to the lower amount of adhesive sites in the

RGD/YIGSR gels, which may have pushed cells to migrate toward the scaffold fibers, where physical cues favored osteogenic differentiation [119].

These results highlighted the importance of scaffold chemical functionalization in modulating cell fate and demonstrated that the proposed hybrid system provides a tunable and versatile platform for *in vitro* pre-vascularization. Future studies should investigate longer culture periods and *in vivo* implantation to assess vascular network maturation and inosculation capacity.

Several groups have pursued an *in vitro* pre-vascularization approach to form microvascular networks. Some studies reported the use of cell-laden hydrogel only, showing success in capillary-like network formation and integration *in vivo*, but mainly for non-load-bearing applications [120,121]. Other studies proposed different types of hydrogels, such as pectin- [122] or gelatin- [123], or HA-based [124], with AM scaffolds, similarly to the work reported in this thesis. Compared to hydrogel only, these hybrid systems combine the advantages of a load-bearing structure with osteogenic properties and an ECM-like soft matrix. In this context, our work introduced a novel step by highlighting the role of hydrogel chemistry in combination with osteogenic AM scaffolds, while to the best of our knowledge most of the studies rely on single hydrogel modification, with RGD as most common peptide.

Next to *in vitro* static pre-vascularization, other strategies have been explored for BTE applications. For instance, ectopic subcutaneous prefabrication, where scaffolds are implanted in highly vascularized host tissues before being transferred to the defect, promoting rapid inosculation [125–127]. However, this method requires multiple surgeries and extended prevascularization times. Alternatively, bioprinting technologies and sacrificial templating of microchannels have been applied to pattern endothelial cells enabling perfusion right after fabrication [128,129]. Finally, bioreactor-based dynamic cultures were also shown to accelerate endothelial network maturation and osteogenic differentiation by reproducing more physiological-like conditions [130,131]. We believe that a future step of the work described in this thesis could be to implement such a dynamic culture in a perfusion bioreactor to further accelerate network maturation before *in vivo* implantation.

The best solution for mandibular bone regeneration: trade-offs towards clinical translation

The overarching goal of this thesis was to develop AM scaffolds for mandibular bone regeneration that could promote both osteogenesis and angiogenesis; one process cannot exist without the other during bone regeneration. Our findings emphasized the complexity of these two aspects, both influenced by many parameters. Thus, identifying one unique

solution is not straightforward, as each approach presents advantages, limitations, and thus the need for trade-offs.

In this work we explored two main approaches: (i) modulating scaffold intrinsic properties (e.g. material and geometry), without additional biological factors, and (ii) *in vitro* pre-vascularization to accelerate osteogenic and angiogenic processes. The first strategy offers advantages in terms of reproducibility, standardization, and potentially easier clinical translation, but it relies on the host's capacity to infiltrate the scaffold and regenerate new bone. On the other hand, pre-vascularization allows for faster integration and can be implemented with autologous cells, but requires additional culture steps, higher technical complexity, and reduced scalability. We believe that the choice between these strategies for a novel regenerative therapy requires a balance between simplicity and efficacy.

This balance has also important implications from a regulatory perspective. Approaches based on intrinsic scaffold properties (pore geometries, biomaterial or surface coating) could be classified as medical devices, similarly to the already approved and commercialized AM scaffolds. In this case, their clinical translation could be faster as they would have to fulfil less requirements such as safety, biocompatibility and sterility. In contrast, pre-vascularization with cells or scaffolds loaded with drugs could fall into the advanced therapy medicinal products (ATMPs) category. The translation would be slower and more expensive, to prove not only safety, but also efficacy, good manufacturing practice (GMP) compliance and long-term follow-up. In our opinion, both avenues hold value and do not represent an either-or choice but rather a sequential solution, where scaffold-based medical devices could be implemented first, probably still in combination with autologous materials, while research on the ATMPs progressively develop toward a final solution.

Future directions for engineered scaffolds

After evaluating all results described in this thesis in terms of osteogenic differentiation and angiogenesis in relation to Chapters 3-6, we identified solutions that could be worth exploring in the future. To gain more insights on all the strategies we tested, *in vivo* studies in relevant animal models should be performed. We made recommendations below to design potential constructs for future studies.

We identified pore geometry as one of the key design parameters to modulate biological response. Notably, the diamond pore architecture stood out by promoting greater osteogenic gene expression while also providing enhanced mechanical properties. For these reasons, we believe that diamond geometry could be a good candidate for future translation *in vivo*. On the material side, we made composites with two potentially

interesting materials for osteogenesis and angiogenesis, TCP and BG. Despite both showing good results, we believe that bioactive glasses represent a more promising candidate because of their combined osteogenic and angiogenic capacity. This multifunctionality makes them more versatile than TCP. In a future study, scaffolds with diamond pore geometry comprising PCL and high content of BGs could be fabricated with light-based printing technologies and assessed *in vivo*. To overcome the limitation of the initial slow response, scaffolds could be filled with autologous adipose tissue or cells harvested from the patient during surgery.

Another interesting study would be to further optimize the ELR coating. For instance, incorporating nanoparticles into the coating could enable a dual release system, where part of the small molecule is immediately released to trigger early response, while the nanoparticles provide a sustained release over time. This solution could be tested in combination with diamond scaffolds and bioactive glasses.

Finally, new molecules could be explored in the functionalization of the hybrid scaffolds for *in vitro* pre-vascularization. For example, in preliminary experiments (not reported in this thesis) we investigated the coupling of nanobodies (e.g. VHH fragments targeting VEGF-R1 or BMP2) to HA hydrogels. These nanobodies could serve a dual purpose: directly activating cells through ligand-receptor binding, or capturing endogenous growth factors secreted by cells, retaining them within the scaffold to locally enhance angiogenesis and osteogenesis. In parallel, hybrid scaffolds could be explored not only for *in vitro* pre-vascularization, but also as a cell-free implant. Here, a controlled release of angiogenic molecules from the hydrogel could be investigated to assess whether this would be sufficient to achieve vascularization without prior cell encapsulation.

Taken together, this thesis proposed multiple strategies to engineer AM scaffolds able to promote osteogenesis and angiogenesis. While several challenges need to still be addressed to bring these implants to the clinic, we believe that this work provides a foundation towards more advanced scaffolds for successful bone regeneration.

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CHAPTER 8

Impact

Craniofacial bone defects are challenging to reconstruct, because restoring function and appearance are crucial. The current treatment, the autologous bone graft, faces significant limitations (see chapter 1 & 7). To overcome these, the development of additive manufactured (AM) scaffolds offers an innovative approach, that can be patient-specific and address clinical needs. In the work described in this thesis, we investigated different approaches to improve bone formation and vascularization, two key aspects that are currently limiting the clinical translation of the scaffolds. The impact of our work extends to different areas, contributing to societal progress, scientific and economic benefits.

Societal impact

The societal impact of this thesis is connected to translational potential of newly developed AM scaffolds. Head and neck cancers represent a significant global burden, with around 400,000 new cases every year involving the oral cavity [1]. Surgical resections often result in complex facial bone defects that require reconstructions with graft material. Autologous bone grafts are associated with donor site morbidity and limited availability of the tissue that can be harvested. Commercial products, such as Bio-Oss®, MagnetOs® or Bonalive®, are already clinically used as alternative, yet their use is mostly limited to dental applications or small non-critical defects (e.g. < 5mm in rat calvarial defect, ridge augmentation [2,3], socket preservation [4]). The inability to restore large complex defects represents thus an unmet societal need where tissue engineering could fulfill a role.

Successful implementation of engineered scaffolds into clinical practice would significantly improve patient care and quality of life by allowing more effective and less invasive defect treatment. For patients whose lives are already affected by cancer treatment and resections, scaffolds could reduce surgical complexity and side effects, reduce post-operative care and provide more natural reconstruction. Moreover, a tissue engineering approach offers the possibility of tailoring the implant design specifically to patient's defects. This aligns with the emerging shift toward personalized and precision medicine, supporting faster recovery. Beyond direct impact in patients' lives, clinical translation of engineered scaffolds would also strengthen awareness and public trust in new therapeutical approaches proposed by tissue engineering by demonstrating tangible progress.

The research performed in this thesis was conducted having clinical application in mind. In this regard, as a first step toward clinical translation, we have already obtained the ethical approval to initiate *in vivo* testing in a porcine bone defect model. These upcoming studies represent an important step toward validating the regenerative capacity of our proposed approaches in a clinically relevant model.

Scientific impact

In the last decade the number of publications revolving around scaffold-based solutions for craniomaxillofacial application has significantly increased (**Figure 1**). As we highlighted in our review of the literature [5], many studies reached the pre-clinical phase. However, we also identified challenges that the field is still facing and that we tried to address in this thesis. Among the three components of bone tissue engineering (i.e. cells, scaffolds and bioactive factors), this thesis focused on scaffold fabrication, ranging from biomaterials to biofabrication. Specifically, we aimed at advancing synthetic scaffolds biological performance by enhancing their osteogenic and angiogenic properties. We investigated multiple strategies including pore geometry, bioactive materials, surface coatings, and pre-vascularization. Collectively, these approaches contributed to advancing fundamental knowledge, methodologies and translation potential.

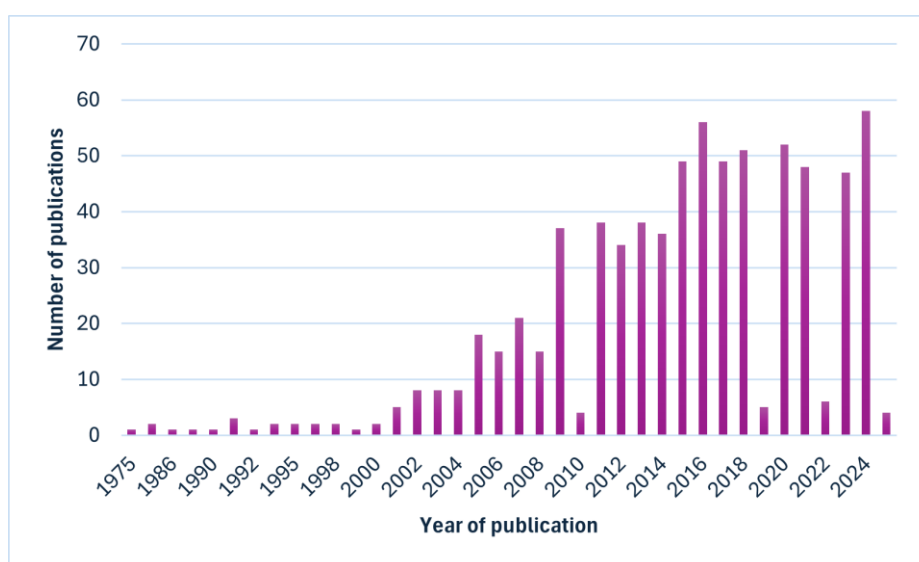


Figure 1. Scientific publications on scaffolds for craniomaxillofacial regeneration since 1975. PubMed, August 2025.

In **Chapter 3** we investigated two non-conventional pore geometries to enhance the osteogenic potential of polycaprolactone (PCL)/ β -tricalcium phosphate scaffolds (β -TCP). We demonstrated that pore geometries act synergistically with bioactive materials in promoting osteogenic differentiation. Importantly, this chapter established a methodological baseline for the remainder of the thesis, where complex geometries were adopted in combination with other biomaterial choices, proposing an alternative to the more conventional woodpile structures. We believe that our thesis will encourage future researchers to focus more on non-conventional and biomimetic pore shapes as default.

In **Chapter 3** and **Chapter 4**, we highlighted the importance of incorporating bioactive ceramics in the scaffolds to enhance both osteogenesis and angiogenesis. In the first study, we produced PCL/ β -TCP scaffolds, and we identified the critical role of surface etching

treatment to enable effective ion release. We realized that particle coverage after scaffold fabrication is often overlooked in the field. Our findings demonstrated that without surface modification, the bioactivity of the scaffolds were hindered. This conclusion was strengthened in Chapter 4 where we fabricated PCL/Bioactive glass scaffolds and treated them with NaOH etching. Furthermore, in this chapter we introduced two novel bioactive glasses, extracted from specific sand layers, with osteogenic, angiogenic and immunomodulatory properties, which could be further investigated with *in vivo* studies.

In **Chapter 5**, we explored for the first time the use of an elastin-like recombinamer (ELR) coating, as delivery platform for small hypoxia-inducing molecules, deferoxamine and phenanthroline. To the best of our knowledge, this was also the first time that phenanthroline was used in combination with 3D osteogenic scaffolds, as usually its use remained confined to 2D applications. The results demonstrated significant pro-angiogenic and osteogenic effects of this molecule that could be further explored in the future for 3D bone applications.

In **Chapter 6**, we applied two well-established chemical modifications within a single system, modifying a hyaluronic acid backbone, enabling to independently control peptide functionalization and crosslinking. Commonly, only one addressable group is introduced on a polymer to conjugate ligands and induce crosslinking. We introduced an accessible methodology to produce polysaccharide materials (e.g. alginate or hyaluronic acid) with two different addressable groups via simple reactions (carboxylic acid activation via DMTMM). We applied this methodology to tune hydrogel properties for modulation of endothelial microvascular organization and osteogenic commitment. Our results paved the way for future studies, where our platform could be implemented in longer culture studies or *in vivo* applications, or for different tissue engineering applications (e.g. osteochondral unit).

Clinical translation of the strategies developed in this thesis would require additional steps to align fabrication and testing to the current regulatory framework. From a regulatory perspective, scaffolds developed in Chapter 3 and 4 could be classified as Class III medical devices, while approaches reported in Chapter 5 and 6 fall into the Advanced Therapy Medicinal Product (ATMP) category due to the presence of cells or the delivery of bioactive molecules. Thus, for example, *in vitro* biocompatibility testing according to ISO 10993 and mechanical testing following ASTM standards are required. For approval of ATMPs, validation of safety and efficacy is required, by performing in preclinical and clinical studies following Good Manufacturing Practice (GMP) conditions. In conclusion, following these quality standards in combination with the novelties introduced by this thesis would bring scaffold-based solutions closer to clinical application.

Economic impact

The economic impact of this research involves multiple aspects, from healthcare cost savings to opportunities for industrial innovation and market development. From a clinical perspective, personalized additive manufactured scaffolds represent a cost-effective alternative to the current gold-standard surgeries, such as the fibula flap transplantation. A multicentric study has estimated that the average cost of fibula flap surgery is around 34,000€, with 30-70% of it attributed to hospital bed costs [6]. In addition, operating room costs have been approximated at 10€ per minute [7]. By using additive manufactured (AM) scaffolds in combination with CAD/CAM technologies that allow accurate surgical planning [8], the surgical time could be reduced by eliminating the need for a secondary donor site, and the hospital stay could also be minimized. Thus, we hypothesize that the cost of the surgery could already be halved, reducing the economic burden on healthcare system.

At the industrial level, our research opens opportunities for innovation and partnerships with the expanding additive manufacturing and biomaterials market. The proposed strategies could also be pushed beyond the craniomaxillofacial applications, to orthopedics and dentistry, broadening their commercial potential. The craniomaxillofacial sector is already characterized by strong industrial activity, with companies such as Stryker (VSP® Reconstruction, Facial iD®), Materialise (Mimics®), involved in virtual surgical planning, or KLS Martin that provides patient-specific implants (IPS®). This work contributed to this innovation pipeline, offering potential new solutions that could be implemented by existing companies or by future spin-offs, accelerating the translation of engineered scaffolds into clinical practice.

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